Integrated Master in Bioengineering

Degradation of 5-Fluorouracil in waters

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of

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Abstract

Cytostatics drugs are a class of pharmaceuticals compounds that are increasingly used in cancer therapies. 5-Fluorouracil (5-Fu) is one of the most commonly used cytostatic drugs worldwide. The concern about cytostatic anticancer drugs in the environment is increasing mainly due to the lack of knowledge about the fate and the impact of these compounds in the water cycle and because such substances can have carcinogenic, mutagenic, teratogenic, genotoxic, and cytotoxic effects, even at low concentrations. As the traditional water and wastewater treatments are not able to remove 5-Fu from the water, different treatment options should be considered. In this work, Fenton oxidation, direct photolysis, photodegradation with hydrogen peroxide and photo-Fenton oxidation were studied for 5-Fu degradation. In order to evaluate the performance of the degradation processes an analytical methodology for HPLC-DAD was developed.

The best performance for the analytical method was obtained at 25 °C, in isocratic conditions (0.2 mL min^{-1}) and with the following mobile phase: 97% water acidified with 0.01% of formic acid (v/v) and 3% methanol acidified with 0.01% of formic acid (v/v). The limit of detection (LOD) was 0.006 mg L^{-1} and the limit of quantification (LOQ) was 0.02 mg L^{-1}.

5-Fu was completely degraded for all treatments but just photo-Fenton oxidation and photodegradation with hydrogen peroxide are able to mineralize the parent compound. The photo-Fenton oxidation and photo-assisted degradation with hydrogen peroxide were the fastest processes, followed by Fenton oxidation and direct photolysis. The photo-Fenton process was the most efficient one with the total mineralization of 5-Fu after 15 minutes of reaction only. During the reaction, different transformation products were detected in HPLC-DAD. Toxicity assays showed a decrease in the toxicity after 8 hours of direct photolysis.

Keywords: 5-Fluorouracil, advanced oxidation processes, Fenton's reagent, photo-Fenton, photolysis, photodegradation, HPLC-DAD

Resumo

Os citostáticos são uma classe de compostos farmacêuticos utilizados no tratamento do cancro; de entre os citostáticos o 5-Fluorouracil (5-Fu) é o mais utilizado. A preocupação com o impacto ambiental dos citostáticos tem vindo a aumentar devido à falta de conhecimento sobre o destino e o impacto destes compostos no ciclo da água e por estes apresentarem múltiplos efeitos adversos, nomeadamente por serem compostos cancerígenos, mutagénicos, teratogénicos e genotóxicos, mesmo em baixas concentrações. Assim sendo, e uma vez que os processos tradicionalmente utilizados no tratamento da água e das águas residuais não são capazes de degradar o 5-Fu, é necessário estudar novos tipos de processos que permitam a eliminação deste composto. Neste trabalho, a degradação do 5-Fu foi estudada utilizando a oxidação com reagente de Fenton, a fotólise direta, a fotodegradação com peróxido de hidrogénio e oxidação por foto-Fenton. A fim de avaliar o desempenho dos processos de degradação foi desenvolvida uma metodologia analítica em HPLC-DAD.

O melhor desempenho para o método analítico foi obtido a 25 °C, em condições isocráticas (0,2 mL min^{-1}) e com a seguinte fase móvel: 97% de água acidificada com 0,01% de ácido fórmico (v/v) e 3% de metanol acidificado com 0,01% de ácido fórmico (v/v). O limite de deteção (LOD) foi de 0,006 mg L^{-1} e o limite de quantificação (LOQ) foi de 0,02 mg L^{-1}.

Todos os processos utilizados permitiram a remoção do 5-Fu abaixo do limite de deteção, mas apenas foto-Fenton e fotodegradação com peróxido de hidrogénio são capazes de mineralizar o composto. Considerado a mineralização, os melhores resultados foram obtidos para a o foto-Fenton, através do qual foi possível obter uma mineralização de 100% após 15 minutos de reação. Durante a reação diferentes produtos de degradação foram detetados no HPLC-DAD. No que diz respeito à toxicidade, os resultados mostraram que ocorre uma diminuição da toxicidade após 8 horas na degradação através de fotólise direta.

Palavras chave: 5-Fluorouracil, processos de oxidação avançada, reagente de Fenton, foto-Fenton, fotocatálise, HPLC-DAD

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Nomenclature

Abbreviations

5-Fu	5-Fluorouracil
ACN	Acetonitrile
AOPs	Advanced oxidation processes
ATC	Anatomical Therapeutic Classification
BCF	Bioconcentration factor
COD	Chemical oxygen demand
DAD	Diode array detector
DOM	Dissolved organic matter
EI	Electron impact or electron ionization
ESI	Electrospray ionization
FA	Fluvic acid
GC	Gas chromatography
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
IC	Inorganic carbon
LC	Liquid chromatography
LOD	Limit of quantification
LOQ	Limit of quantification
MeOH	Methanol
MRM	Multiple reaction monitoring
MS	Mass spectrometry
NCI	Negative chemical ionization
PDA	Photodiode array detector
QqQ	Triple quadrupole
QqToF	Quadrupole orthogonal acceleration time-of-flight mass spectrometer
RI	Refractive index
RP	Reversed-phase
RSD	Relative standard deviation
TC	Total carbon
TOC	Total organic carbon
UPLC	Ultra performance liquid chromatography
UV	Ultraviolet
WWTPs	Wastewater treatment plants

Notation

K_{OC}	Organic carbon partition coefficient	
K_{OW}	Octanol-water partition coefficient	
pKa	Dissociation constant	
R	Correlation coefficient	
T	Temperature	°C
t	Time	min or h
$t_{1/2}$	Half-life	min or h
t_M	Migration time	min or h
t_R	Retention times	min or h

1 Introduction

Pharmaceutical drugs are a large and diverse group of medicinal compounds for human and veterinary health that are used in very high amounts throughout the world [1-4]. Since the mid-1990s pharmaceutical drugs have emerged as a novel class of water contaminants [5]. Their occurrence in aquatic and terrestrial environments, although often detected at trace levels (sub-μg L^{-1}), resulted in emerging concerns in public and regulatory agencies [6], because of the potential impact on human health and on the environment as their environmental degradation is lower than the environmental input rates, resulting in accumulation throughout the years [4, 5].

Among various classes of pharmaceuticals, cytostatic chemotherapy drugs are of particular concern because they are potentially carcinogenic, mutagenic, genotoxic, cytotoxic, and teratogenic, even at low concentrations [7, 8]. Over the last decade the number of cytostatics used in cancer chemotherapy has increased considerably, about 10% per year in developed countries [2].

The World Health Organization classified cytostatic drugs as Antineoplastic and Immunomodulating Agents (Class L) [3]. Moreover, the Anatomical Therapeutic Classification (ATC) scheme divides cytostatics into five classes according to their mode of action in alkylating agents, antimetabolites, plant alkaloids and other natural products, cytotoxic antibiotics and related substances, and other antineoplastic agents [9]. In Figure 1.1 are presented commonly-used cytostatics drugs divided according to ATC classification.

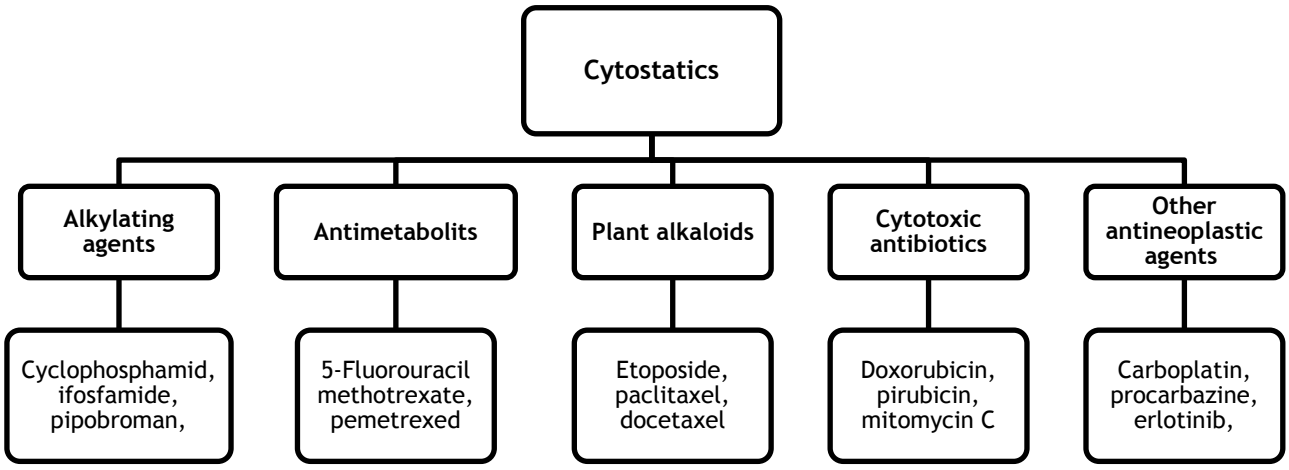
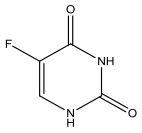


***Figure 1.1** Cytostatics divided according to ATC classification [3].*

1.1 5-Fluorouracil

5-Fluorouracil (5-Fu) belongs to the class of antimetabolites and it is the most commonly and widely used cytostatic in the world [9, 10]. 5-Fu is a fluoropyrimidine rationally designed by Duschinsky et al.[11] as a targeted anticancer drug, synthesized and patented in 1957 and immediately introduced in the clinic activities [12, 13]. 5-Fu exerts its anticancer effects through inhibition of thymidylate synthase and incorporation of its metabolites into RNA and DNA [10]. 5-Fu can be administered orally, topically or parenterally. 5-Fu is not completely metabolized being 10%-30% excreted in the parental form into hospital or municipal wastewater [14]. According to the International Agency for Research on Cancer, the concentration threshold for toxicity (carcinogenicity) of 5-Fu is 230 ng L^{-1} [15]. 5-Fu structure, its elemental formula, molecular weight and classification according to the ATC are presented in Table 1.1 [10].

Table 1.1 *Structure of 5-Fu, its elemental formula, molecular weight and classification according to the ATC (adapted from: [10])*

Cytostatic/chemical name/CAS	Elemental formula	Monoisotopic mol. mass (g mol^{-1})	Chemical structure	Group
5-Fu/5-fluoro-1*h*-pyrimidine-2,4-dione/51-21-8	$C_4H_3FN_2O_2$	130.017		Antimetabolic agent: pyrimidine analogue

The main physical-chemical properties of 5-Fu, including dissociation constant (pKa), bioconcentration factor (BCF), octanol-water partition coefficient (K_{OW}), organic carbon partition coefficient (K_{OC}), atmospheric $HO^{\bullet}$ rate, solubility in water at 22 °C, Henry's coefficient (K_H) and vapour pressure are presented in

Table 1.2; such properties determine its occurrence in the environment. In fact 5-Fu is a weak acid (pK_a 8) and an extremely polar compound with a log K_{OW} of -1.0. 5-Fu is mainly distributed in liquid and solid phases due to its low value of the Henry's coefficient and its extremely low value of vapour pressure, and thereby the fraction removed by volatilization can be considered negligible [10]. Octanol-water partition coefficient and organic carbon partition coefficient can be used to determine the sorption and affinity of a given substance to organic matter; the low K_{OW} suggests that 5-Fu presents low adsorption to suspended solids in water and the K_{OC} suggests that 5-Fu has high mobility in soil/sediments [3]. Studies have shown that the sorption of 5-Fu on sludge in biodegradation treatments is negligible [10]. 5-Fu BCF suggests that the potential for bioconcentration in aquatic organisms is low. The

atmospheric HO• rate describes the reaction rate of a compound with hydroxyl radicals (HO•); compounds with high atmospheric HO• rate constants have great potential for being degraded through advanced oxidation processes (AOPs). The high value of 5-Fu' atmospheric HO• suggests that it has a great potential for being oxidized by AOPs [10]. 5-Fu is light sensitive in solution with an UV maximum-absorbance peak at 266 nm.

Table 1.2 *Physical-chemical properties of 5-Fu (adapted from [3, 10])*

pKa	BCF	log K_{OW}	K_{OC}	Atmospheric HO• rate (10^{-9} cm^3 $Molecule^{-1}$ s^{-1})	Solubility ($mg\ L^{-1}$)	K_H ($atm\ m^3\ mol^{-1}$)	Vapour pressure (mm Hg)
8.0	3.6	-1.0	8	58.3	1.11×10^{4}	1.66×10^{-10}	2.68×10^{-6}

Cytostatics have been accumulating in waters along the years due to the increasing demand for chemotherapy drugs and their inefficient removal in wastewater treatment plants (WWTPs) [7]. 5-Fu has been detected in concentrations ranging from below 0.0009 to 122 $\mu g\ L^{-1}$ in hospital effluents and in concentrations ranging from 5.0 to 47 $ng\ L^{-1}$ in municipal wastewater, Table 1.3.

Table 1.3 *Occurrence of 5-Fu in waters*

Matrix	Concentration range µg L	Ref.
Hospital effluent	< 0.0009 - 0.038	[16]
Municipal wastewater	< 0.005 - 0.027^{-1}	[17]
Hospital effluent	20 - 122	[17]
Hospital effluent	0.09 - 4.0^{-1}	[18]
Hospital wastewater (oncological ward)	0.035 - 0.092	[9]
Municipal wastewater	0.014 - 0.047	[9]

Although the amount of 5-Fu in the aquatic environment may be still considered low, its continue input may constitute a long-term potential risk for aquatic and terrestrial organisms. Pharmaceutical compounds released in the environment and in particular in the aquatic medium constitute a serious environmental problem because these compounds are extremely resistant to biological degradation processes and usually escape intact from conventional treatments plants, and may impose serious toxic and others effects to human and living organisms. Therefore it is important to study the application of non-biological processes for the destruction of pharmaceuticals in waters with emphasis in AOPs [1]. AOPs represent an interesting alternative, since they can be employed in association with biological treatment

for wastewater remediation, as a pre-treatment, increasing the biodegradability, or as post-treatment for the degradation of persistent compounds [19].

AOPs are broadly defined as oxidation methods characterized by the production and use of hydroxyl radicals ($HO^{\bullet}$) for the oxidation of chemical substances (Eq. 1) [1].

$$AOPs \rightarrow HO^{\bullet} + pollutant \rightarrow \cdots \rightarrow CO_2 + H_2O + inorganic\ ions \qquad \textbf{Eq. 1}$$

AOPs can be classified as heterogeneous or homogeneous; both categories can be subdivided according to the need of energy use, Figure 1.2 [20]. The main advantages of AOPs are that they operate in most cases at near ambient temperature and pressure and generate strongly oxidizing radical species for nearly complete decomposition of organic pollutants [21].

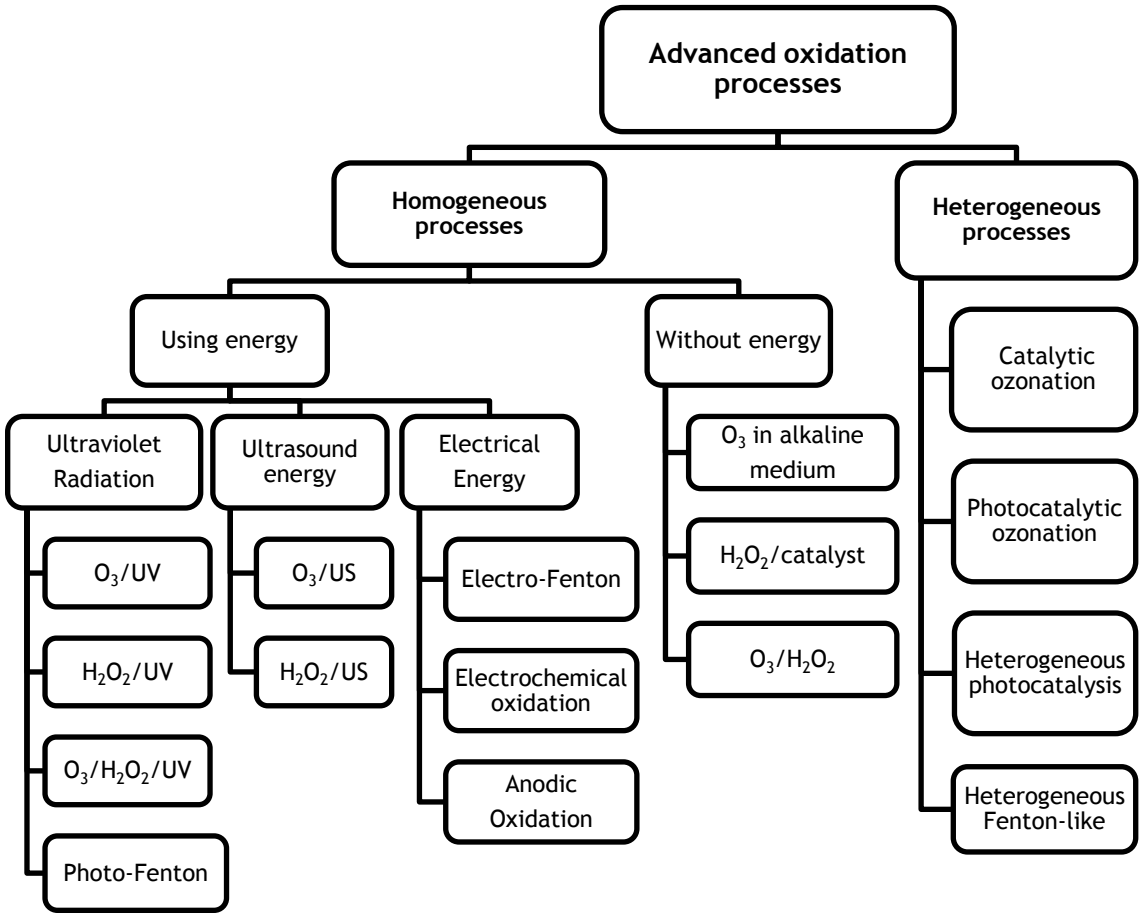


Figure 1.2 *Advanced oxidation processes classification (adapted from [20]).*

1.2 Analytical method for 5-Fu in waters

An analytical method for 5-Fu analysis in water is a tool necessary for any studies requiring the concentration measurements. However, the method for 5-Fu analysis faces three main challenges: 1) the analyte is very polar, thus its analysis is generally regarded as difficult; 2) it is only found at trace levels in the environmental; and 3) the composition of the matrix, usually wastewater, interferes with the analysis [16].

There are several methods described in the literature for the analysis of 5-Fu in water matrix: gas chromatography/mass spectrometry (GC/MS) [9, 15, 22, 23], high performance liquid chromatography (HPLC) [8, 16, 24-33], and ultra performance liquid chromatography (UPLC) (Table A 1-Appendix A) [9].

1.2.1 Chromatography

Chromatography is a physical method used for the separation of components of a sample, in which the components are distributed between two phases one of which is stationary (stationary phase) while the other (the mobile phase) percolates through it in a definite direction. The chromatographic process occurs as a result of repeated sorption/desorption during the movement of the sample components along the stationary bed, the separation occurring due to differences in the distribution constants of the individual sample components [34, 35].

A distinction between the principal chromatographic methods can be made in terms of the properties of the mobile phase. If the mobile fluid is a gas the technique is known as gas chromatography; if it is a liquid the technique is called liquid chromatography. Each technique may be further sub-divided according to the nature of the stationary phase [34-36].

High performance liquid chromatography is an important qualitative and quantitative technique, used for the studied of various chemical compounds. In HPLC the stationary phase is typically in the form of a stainless-steel column packed with extremely small porous silica-particles embedded in liquid stationary phases (most common C18), and the liquid mobile phase (or eluent) is pumped through the column at elevated pressure. The sample to be analyzed is retarded by specific chemical or physical interactions with the stationary phase. The retardation depends on the nature of the analyte and composition of both stationary and mobile phases [36, 37]. The eluted molecules differ from the mobile phase components by certain physicochemical properties such as UV absorption, which make them detectable. An electrical signal is associated with molecular detection, and the graphic output of this signal is known as a chromatogram [37].

The separated components of a mixture eluting at different retention times (t_R) are displayed as peaks in the chromatogram. Different peaks on the chromatogram belong to different components of the separated mixture. The peaks in the chromatogram may have different heights (and peak areas) depending on factors such as the amount of compound in the mixture, amount of sample injected, and sensitivity of the detection procedure [36].

A variety of HPLC types have been described in the literature, the differentiation being based on various criteria such as the nature of the stationary and mobile phases, the type of interactions assumed to lead to the separation and the range of concentration of specific solvents in the mobile phase [36].

Reversed-phase HPLC (RP-HPLC) is the most commonly used form of HPLC, and a very large number of compounds can be separated by RP-HPLC. The separation is based on analytes' partition coefficients between a polar mobile phase and a hydrophobic (nonpolar) stationary phase. A wide variety of nonpolar stationary phases is available. The stationary phase for RP-HPLC can be obtained, for example, by chemically bonding long hydrocarbon chains on a solid surface such as silica. The most common chain bound to silica is octadecyl (C18, as it contains 18 carbon atoms), which has high hydrophobic character. RP-HPLC typically uses a polar mobile phase such as a mixture of an organic solvent (methanol, for example) and water. Small amounts of buffers can also be added to the mobile phase in RP-HPLC. The mechanism of separation is primarily attributed to solvophobic or hydrophobic interactions. The retention of the analyte on the stationary phase is dependent on the contact surface area between the nonpolar moiety of the analyte molecule and the stationary phase. For this reason RP-HPLC is especially suitable for the separation of analytes with a large hydrophobic surface area (and usually with a large log K_{ow}). In RP-HPLC the separation is typically considered to be based on the partition of the analyte between the stationary phase and the mobile phase, although some experiments can be explained by adsorption equilibrium [36, 37].

A typical HPLC system includes: (1) a solvent delivery system that provides the solvent(s) necessary as a mobile phase for the HPLC; (2) a high-pressure pumping system that consists of pump(s) able to deliver a constant flow of solvent through the injector, chromatographic column, and through the detector(s); (3) an injector, which role is to add in the mobile phase a small, precisely measured volume of a solution containing the sample (the injection must be done reproducibly and accurately); (4) a chromatographic column (possibly with a guard column or precolumn) designed for performing the separation in HPLC; (5) one or more detectors and (6) a controller/data processing unit, Figure 1.3 [36].

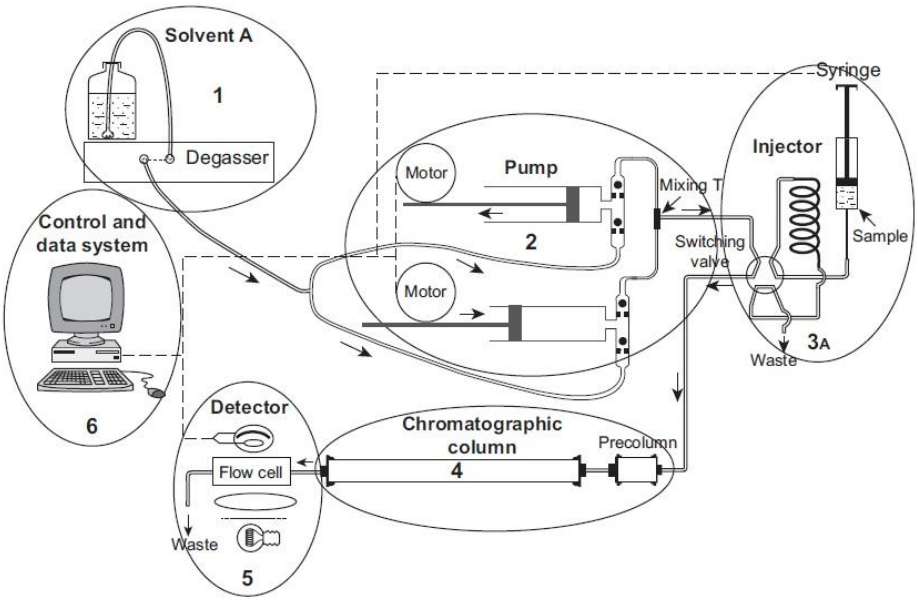


Figure 1.3 *Schematic description of a simple HPLC system: 1) a solvent supply system, 2) a high pressure pumping system, 3) an injector, 4) a chromatographic column, 5) detectors, 6) a controller/data processing (adapted from [36]).*

The detection of the molecular species eluted from the chromatographic column can be done using a variety of principles and techniques. The main types of detectors used in HPLC are refractive index (RI), ultraviolet (UV-Vis) and fluorescence, but there are also diode array, electrochemical and conductivity detectors. The qualities of the detectors should include sensitivity, reproducibility of the response, linearity in a wide range of concentrations of the sample, capability for detection in a small volume of sample, capability of not contributing to peak broadening, and stability to changes in flow and environmental parameters [37].

A diode array detector also known as a photodiode array detector (PDA), provides UV spectra of eluting peaks while functioning as a multi wavelength UV/Vis absorbance detector. It facilitates peak identification and the preferred detector for method development. An advantage to diode array HPLC detectors is the ability to select the best wavelength for analysis [37].

High performance liquid chromatography with ultra-violet detection (HPLC-UV) is the most used method reported in the current literature. This technique appears to be the most feasible for attaining maximal sensitivity (a lower limit of detection). The main advantage of HPLC when compared to GC is that HPLC does not require the high volatility and thermal stability of the analytes or their conversion to their derivatized forms [10]. HPLC is most commonly coupled to UV detection; however, HPLC in combination with diode array detector (HPLC-DAD) has been broadly used in determination of organic pollutants and was preferred as a routine detection method. UV/VIS diode array detectors for HPLC improve the selectivity

of this technique to the extent that it can give multiple data about each peak, allowing peak identification through retention time and spectral matching [38].

1.3 Degradation processes

Conventional biological, physical, and chemical processes have been employed to remove 5-Fu from waters. The main methods used for 5-Fu degradation are:

a. Biodegradation;
b. Ozonation;
c. Chlorination and Bromination;
d. Photolysis;
e. Heterogeneous photocatalysis;
f. Photo-Fenton.

Biodegradation is the most studied method to remove 5-Fu from contaminated water [39]. More recently, advanced oxidation processes (namely photolysis, heterogeneous photocatalysis, ozonation and photo-Fenton), as well as chlorination and bromination, have been studied for the removal of 5-Fu from water.

1.3.1 Biodegradation

Biodegradation is a widely used process to remove organic contaminants in wastewater treatment [3]. However, the biodegradability of cytostatics depends on their chemical structure and stereochemistry. The major problems related with biodegradation of cytostatics are the following: many of these compounds are hydrophilic and will not readily sorb to the sludge; some of the molecules contain halogen, which is known to be problematic for the biodegradation; and many could be toxic to microorganisms [10]. 5-Fu does not contain a sugar linked to pyrimidine as a part of its molecular structure that might influence its biodegradability [39]. Moreover, 5-Fu has been reported to be toxic against Gram-positive bacteria, which build the main group in sewage treatment plants [40].

Biodegradability data available from the literature show different degradability for 5-Fu obtained at different conditions, and reveal contradictory results regarding the potential for biodegradation of 5-Fu. While the results of Kiffermeyer *et al* (1998) [8], Mahink *et al* (2007) [41], and Kosjek *et al* (2013) [9] show degradation for 5-Fu above 90% within the tested conditions, results of Yu *et al* 2006 [42] show only partial degradation, Table 1.4. At high

concentrations on experiments performed by Kümmerer et al (1997) [39], no degradation was reported at all within 28 days.

***Table 1.4** Biodegradability studies of 5-Fu*

	5-Fu (mg/L)	t (days)	Fate	Analysis	Matrix	Ref.
Closed bottle test	9	28	not biodegraded	O_2 with oxygen electrode	Mineral medium in deionised water	[39]
Zahn-Wellens test	854 (175)	2	2% biodegraded	DOC	Mineral medium in deionised water (effluent from communal hospital)	[39]
OECD confirmatory test	5	2	92% biodegraded	HPLC-DAD	Synthetic sewage	[8]
Aerobic batch biodegradation	0.001	50	50% biodegraded	GC-MS	1:1000 diluted waste activated sludge	[42]
Elimination by activated sludge	0.005	1	>95% biodegraded	LSC ([2-^{14}C]5-Fu)	Municipal wastewater from local sewer 8-18 g L^{-1} suspended solids	[41]
Batch biodegradation	1	2	>99%	LC-MS/MS	Wastewater	[9]

The contradictory results on biodegradability of 5-Fu reported in the literature can be explained by the fact that most of the experiments were conducted in the concentration range 9-854 mg L^{-1}; at high concentrations 5-Fu is cytotoxic to microorganisms. The high concentrations have had cytotoxic effects on the degrading microorganisms, potentially leading to false negative results [43].

1.3.2 Ozonation

The application of ozone in drinking-water treatment is extensively used around the world [44]. Ozone (O_3) is a powerful oxidant that decomposes in water to form $HO^{\bullet}$, which are stronger oxidizing agents [1]. The elimination of organic contaminants by ozone treatment can thus occur by two different reaction pathways: direct reaction with molecular ozone and indirect reaction with free radicals, mainly hydroxyl radicals, generated by the decomposition of ozone [3], namely by a catalytic way. In addition to ozonation via molecular O_3 (direction reaction) and via hydroxyl radical (indirect reaction), other possibilities of ozone application in wastewater treatment are available. Ozone can be used in conjugation with ultraviolet radiation (O_3/UV), hydrogen peroxide (O_3/H_2O_2), or a combination of these (O_3/UV/H_2O_2), in addition to O_3/ultrasound and O_3/TiO_2. These techniques increase the generation of hydroxyl radicals [20].

5-Fu is a nitrogen base, related to nucleobases. It is known that ozone reacts rapidly in aqueous medium with nucleobases and so ozonation could be considered as an adequate method to remove 5-Fu from water. Rey et al.[45] studied the ozone inactivation of 5-Fu. Ozone in oxygen (16-18 mg L^{-1}) was bubbled into the samples at a flow rate of 3.3-3.6 L h^{-1} during 60 or 75 min. The results showed that ozonation allowed a removal of 5-Fu bellow the limit of detection in 30 min [45].

However, one of the major drawbacks with ozonation is its cost and the necessity of continuous feeding ozone due to its short half-life, typically being 20 min. Ozone's half-life depends on pollutants present in the aqueous matrices, the presence of salts, pH, and temperature. In alkaline conditions, for example, ozone decomposition is accelerated, and a strict monitoring of the effluent pH is required [46].

1.3.3 Chlorination and Bromination

Chlorination has been widely used in water treatment due to the low cost and high disinfection efficiency of chlorine [47]. Its great advantage is its high reaction rate with numerous inorganic and organic micropollutants present in waters [14].

Bromination reactions have also been receiving interest in the oxidation of water pollutants, especially in waters with high bromide (Br) concentrations [14]. Bromine-containing species tend to react faster with many organic species than their chlorine-containing analogues. Bromide ion (Br^-) occurs at widely varying concentrations in aqueous systems. It is oxidized readily by hypochlorous acid (HOCl) to form hypobromous acid (HOBr) and associated compounds, Eq. 2 to Eq. 3 [14].

$$Cl_2 + H_2O \leftrightarrow HOCl + Cl^- + H^+ \quad \textbf{Eq. 2}$$

$$HOCl + Br^- \rightarrow HOBr + Cl^- \quad \textbf{Eq. 3}$$

$$HOBr \leftrightarrow OBr^- + H^+ \quad \textbf{Eq. 4}$$

$$Br_2 + H_2O \leftrightarrow HOBr + H^+ + Br^- \quad \textbf{Eq. 5}$$

Li et al.[14] studied the kinetics of 5-Fu degradation by chlorine and bromine. 5-Fu is fully degraded in 30 min when 1 mg $L^{-1}$of chlorine and 0.08 mg L^{-1} bromide is present in the water. The proposed pathway for 5-Fu degradation is shown in Figure 1.4. [14]. The pathway suggests that 5-Fu chlorination proceeds via chlorine incorporation at the 6th carbon in the

heterocyclic pyrimidine ring of 5-Fu. Then, the formation of mono- and di-halogenated 5-Fu byproducts occurs, and finally the ring's breakdown [14].

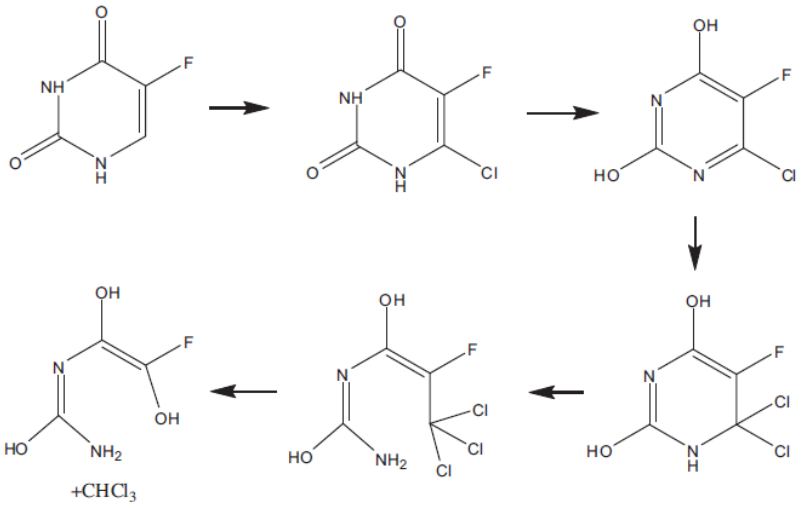


Figure 1.4 *Proposed pathway of 5-Fu degradation by chloride (adapted from:[14]).*

Besides the chlorination and bromination low cost, the advantage of chlorine is related with the fact that it reacts with numerous inorganic and organic micropollutants present in waters; their main drawback is the formation of possible harmful disinfection by-products, such as halogenated organic compounds, some of which exhibit a potentially carcinogenic activity [47].

1.3.4 Photolysis

Photolysis consists in the interaction between light (natural or artificial) and a target molecule, resulting in photochemical reactions that can lead to its degradation. There are some factors that affect the efficiency of photolysis such as the absorbance spectrum, the quantum yield of photolysis and the water matrix. Photodegradation may occur via direct or indirect pathway [1].

Direct photolysis requires absorption of light by the chemical substance of interest and leads to chemical bond cleavage [48].

Lin et al. [48] studied the effect of direct photolysis in the degradation of 5-Fu. The results showed that the half-life ($t_{1/2}$) for 5-Fu was 56 ± 11 h, when the 5-Fu initial concentration ranged between 38 nM to 77 µM. The pH of the solution affected the 5-Fu photolysis; direct photolysis rates were similar at pH 6 and 7 and rates doubled for pHs of 8

to 9. This could be explained by the significant shift of the maximum absorbance of 5-Fu to higher wavelengths for pH 8 and pH 9 [48].

In the indirect photolysis, the light is absorbed by some species present in the chemical environment (not by the target pollutant), and these species initiate a series of reactions that result in the transformation of the target compound [48].

The fate of 5-Fu is greatly affected by the water matrix. Dissolved organic matter (DOM), nitrate (NO_3^-), and bicarbonate (HCO_3^-) are generally present in natural surface water at various concentrations and are the major water constituents affecting photolysis [48]. Lin et al. [48] studied the effect of these compounds in the degradation of 5-Fu. Fluvic acid (FA) was utilized to evaluate the influence of DOM in photolysis. The results showed that FA had a slight effect in the photolysis rate - for a FA concentration of 1 mg L^{-1}, the rate of photolysis is equivalent to the directed photolysis rate, Table 1.5. When FA concentration increases to 5 and 10 mg L^{-1}, half-lives decrease by 8 and 30%, respectively, thus having a positive effect on the degradation rate. The light absorption by FA produces reactive species that react with 5-Fu leading to its degradation. Higher concentration of FA can result in a higher generation of reactive species increasing 5-Fu degradation rate [48].

Table 1.5 *Effect of different water matrix and degradation process on the 5-Fu removal efficiency by photolysis [48]*

Matrix	Degradation process	Rate constant (h^{-1})	$t_{1/2}$ (h)	Removal efficiency (%)
DI	Direct photolysis	0.0109 ± 0.0015	63 ± 8[a]	-
		0.0108 ± 0.0009	64 ± 5[b]	-
		0.0138 ± 0.0012	50 ± 4[c]	-
		0.0220 ± 0.0006	32 ± 1[d]	-
DI+FA	Photo with DOM, O_2, and other DOM-derived radicals	0.0133	52	30
DI + HCO_3^-	Photo with HCO_3^-	0.0399 ± 0.0029	17 ± 1	70
DI + NO_3^-	Photo with $OH^•$	0.0249 ± 0.0015	28 ± 2	67
DI + HCO_3^- + NO_3^-	Photo with $HCO_3^-$and $OH^•$	0.0871	8.0	92

[a]pH 7.0; [b]pH 6.0;[c]pH 8.0;[d]pH 9.0.

The effect of nitrate on photolytic degradation was also investigated by Lin and co-workers [48]. In the presence of nitrate the photolysis rates are improved. Nitrate produces hydroxyl radicals upon irradiation (Eq. 6), thus when the concentration of nitrate increases from 1 to 5 mg L^{-1}, the 5-Fu degradation rates were almost doubled and the photolysis half-lives decrease [48].

Bicarbonate is the most common inorganic anion in the natural environment. Bicarbonate anions can act as hydroxyl radical scavengers, and the reaction of hydroxyl radicals and bicarbonate is the major source of CO_3^{-} in aquatic environment (**Eq. 7**). CO_3^{-} is a more selective oxidant than $HO^{\bullet}$ and undergoes less scavenging by DOM, resulting in a higher steady state concentration compared to $HO^{\bullet}$ [48]. Lin et al.[48] verified that bicarbonate alone reacts with excited states of 5-Fu, thus enhancing direct photolysis rates; consequently, half-life decreases from 17 to 13 hours when the concentration of bicarbonate increases from 1 mM to 10 mM.

In the presence of nitrate and significant quantity of bicarbonate, 5-Fu was rapidly removed through indirect photolysis [48]. One possible explanation for this result is the scavenging of $HO^{\bullet}$ by bicarbonate (Eq. 8). The scavenging of $HO^{\bullet}$ by bicarbonate could prevent $HO^{\bullet}$ and $NO_2^{\bullet}$ recombination, resulting in a higher generation rate of $CO_3^{-\bullet}$. As $CO_3^{-\bullet}$ is a more selective oxidant than $HO^{\bullet}$ the performance of the reaction is improved.

$$NO_3^- + hv \rightarrow HO^{\bullet} \qquad \textbf{Eq. 6}$$

$$HCO_3^- + HO^{\bullet} \rightarrow CO_3^{-\bullet} \qquad \textbf{Eq. 7}$$

$$5 - Fu + CO_3^{-\bullet} \rightarrow byproducts \qquad \textbf{Eq. 8}$$

Kosjek et al. [9] used photodegradation to remove 5-Fu from wastewater. The UV degradation of 5-Fu followed pseudo-first order kinetics with degradation rate constant of 0.045 min^{-1} and a half-life of 15 min. The efficiency of photolysis was improved by adding H_2O_2 into the aqueous solution of 5-Fu. 5-Fu was removed (99.6%) within 10 min by UV/H_2O_2 treatment [9].

1.3.5 Heterogeneous photocatalysis

Heterogeneous photocatalysis is an emerging technique that can be used for water treatment. Heterogeneous semiconductor photocatalysis using titanium dioxide (TiO_2) as photocatalyst has demonstrated to be effective for removing pollutants from water [15, 49].

The principle of photocatalysis is based on the excitation of TiO_2 by light (UV or even visible, depending on the photocatalyst used). Under the action of photons, the semiconductor produces highly oxidizing free radicals allowing the destruction of compounds adsorbed on its surface, as illustrated in Figure 1.5.

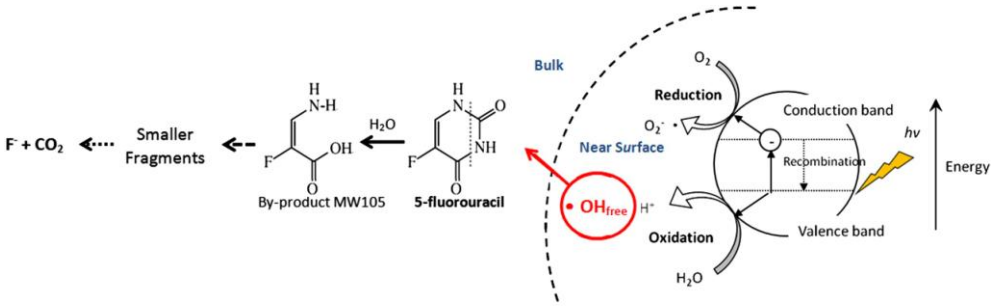


Figure 1.5 *Scheme of the photocatalytic oxidation of 5-Fu (adapted from: [50]).*

Photocatalytic reactions usually involve TiO_2 suspensions with the catalyst loading being an important parameter that affects the performance of the reaction. The light wavelength and intensity, the solution pH, the addition of H_2O_2 and the water matrix also affect the performance of degradation [1], among other process variables (e.g. temperature and initial pollutant concentration).

Lin and Lin [15] studied the effect of catalyst employed (Aldrich-TiO_2, Degussa P25 and ZnO), photocatalyst loading, 5-Fu concentration and the effect of initial pH in the photocatalytic oxidation via UV/TiO_2 in aqueous systems. Under optimized conditions, 5-Fu was completely decomposed within 2 hours, Table 1.6. Despite the rapid parent compound removal, more than 24 hours was required to reach complete mineralization [15].

Table 1.6 *The effect of type of photocatalyst, photocatalyst loading, 5-Fu concentration and initial pH on 5-Fu removal efficiency ([15])*

Test	[5-Fu] μg L^{-1}	Catalyst	[Catalyst] mg L^{-1}	pH	Rate constant (min^{-1})	t (h)	Removal efficiency (%)
Type of photocatalyst employed	50	Aldrich-TiO_2	5	5.8	0.0365	8	98.2
		Degussa P25			0.0096	2	99.9
		ZnO			0.0041	8	87.9
Photocatalyst loading	200	Degussa P25	5	5.8	-	4	75.2
			20		0.0375	1.5	99.9
			50		-	4	95.3
			100		-	4	89.4
5-Fu concentration	20	Degussa P25	20	5.8	-	2	99.9
	50				-	2.5	99.9
	100				-	4	97.6
	200				-	1.5	99.9
Initial pH	200	Degussa P25	20	3	-	4	71.7
				5.8	-	1.5	99.9
				7.8	-	4	80.8
				10	-	4	72.6

Although heterogeneous photocatalysis was demonstrated to be an effective process for 5-Fu degradation, when the environmental impact of heterogeneous photocatalysis was compared with that of Fenton and photo-Fenton reactions, it was concluded that heterogeneous photocatalysis presents a higher environmental impact [51]. Munoz et al [51] compared two possibilities with regard to the advanced oxidation step in a coupled AOP–biological process, heterogeneous semiconductor photocatalysis and homogeneous photo-Fenton, for treatment of industrial wastewater containing α-methyl-phenylglycine: the environment impact was evaluated by means of a life cycle analysis and the authors compared a similar system comprising TiO_2 photocatalysis instead photo-Fenton treatment. The study showed that the wastewater treatment plant based on heterogeneous photocatalysis involves a higher environmental impact than the photo-Fenton alternative, which displays impact scores between 80% and 90% lower in most impact categories assessed.

Degradation of 5-Fu by Fenton and photo-Fenton reactions was not well described in the literature; there is only one article which reports the use of these processes for 5-Fu degradation. Lutterbeck et al. [19] compared the efficiency of three different AOPs (photolysis, photo-Fenton and heterogeneous photocatalysis with TiO_2) and conclude that the highest mineralization degrees were obtained with the photo-Fenton reaction. Thus, dark Fenton and photo-Fenton processes can be a promising alternative for the 5-Fu degradation.

1.4 Fenton oxidation

The Fenton reaction is an AOP which can be effectively used in the degradation of hazard organic pollutants in water via generation of hydroxyl radicals by catalytic decomposition of hydrogen peroxide using ferrous ions (Fe^{2+}) as the catalyst. The Fenton reaction was discovered by H. J. H. Fenton in 1894, but its application as an oxidizing process for destroying toxic organics was not applied until the late 1960s [21, 52]. In the last few decades, Fenton reaction has been employed for the treatment of diverse wastewaters, as for example, olive oil industries, textile industries, paper pulp factories, as well as effluents from refinery and fuel terminals, sludge waste, landfill leached and contaminated solids, among others [20, 21].

The traditionally accepted Fenton mechanism is in a simplified way represented by the Eq. 9 to Eq. 15. Firstly, Fe^{2+} initiates and catalyses the decomposition of H_2O_2, resulting in the generation of $HO^{\bullet}$, Eq. 9 [52, 53].

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + OH^{-} \left(k_1 = 63 - 76\, M^{-1}\, s^{-1}\right) \text{ (chain initiation)} \qquad \textbf{Eq. 9}$$

$HO^{\bullet} + Fe^{2+} \rightarrow Fe^{3+} + OH^{-}\ (k_2 = 4.3 \times 10^8\ M^{-1}\ s^{-1})$ (chain termination) **Eq. 10**

The ferric ions (Fe^{3+}) resulting from Eq. 9 can catalytically decompose H_2O_2 forming ferrous ions and radicals. The reaction of hydrogen peroxide with ferric ions is called Fenton-like reaction. Fenton-like reaction allows Fe^{2+} regeneration in an effective cyclic mechanism (Eq. 11 to Eq. 15) [52, 54].

$Fe^{3+} + H_2O_2 \rightarrow Fe^{III}(HO_2)^{2+} + H^{+}\ (k_3 = 3.1 \times 10^6\ M^{-1}\ s^{-1})$ **Eq. 11**

$Fe^{III}(HO_2)^{2+} \rightarrow Fe^{2+} + HO_2^{\bullet}\ (k_4 = 2.7 \times 10^{-3}\ M^{-1}\ s^{-1})$ **Eq. 12**

$Fe^{2+} + HO_2^{\bullet} \rightarrow Fe^{3+} + HO_2^{-}\ (k_5 = 1.6 \times 10^6\ M^{-1}\ s^{-1})$ **Eq. 13**

$Fe^{3+} + HO_2^{\bullet} \rightarrow Fe^{2+} + O_2 + H^{+}(k_6 = 1.2 \times 10^6\ M^{-1}\ s^{-1})$ **Eq. 14**

$HO^{\bullet} + H_2O_2 \rightarrow H_2O + HO_2^{\bullet}(k_7 = 3.3 \times 10^7\ M^{-1}\ s^{-1})$ **Eq. 15**

In the presence of an organic substrate (R–H), the hydroxyl radical abstracts a hydrogen atom from R–H and generates an organic radical ($R^{\bullet}$), which is highly reactive and can be further oxidized. If the concentration of reactants are not limiting, in principle the organic subtract can be completely converted into carbon dioxide and water [21, 52, 53].

$R - H + OH^{\bullet} \rightarrow H_2O + R^{\bullet} \rightarrow \cdots \rightarrow CO_2 + H_2O\ (k_8 \approx 10^7 - 10^{10}\ M^{-1}\ s^{-1})$ **Eq. 16**

The main advantages of Fenton reactions are the following: Fenton process can be carried out at atmospheric pressure and room temperature, requires reagents that are readily available, easy to store, handle, safe and are environmental friendly; moreover, the high mineralization efficiency of this technology enables the transformation of organic pollutants into non-toxic carbon dioxide (CO_2). The main drawbacks related with the Fenton reactions are: the wastage of oxidants due to the radical scavenging effect of hydrogen peroxide; the continuous loss of iron ions and the formation of solid sludge [54].

1.4.1 Effect of operational parameters

Operating parameters, such as, pH, ferrous ion concentration, hydrogen peroxide loading, initial concentration of pollutants and operating temperature need to be optimized to improve the Fenton reaction performance [55].

1.4.1.1 Effect of the initial pH

Fenton processes are strongly dependent of the solution pH due to the iron speciation factors. An optimum pH for the Fenton reaction was found to be around 3 and the process needs a strict pH control. At pH value 2.8 half of the Fe is present as the complex ion $Fe(OH)^{2+}$ and half as Fe^{3+}. The activity of the Fenton reagent is reduce at lower pH values due to the declining in the concentration of $Fe(OH)^{2+}$, while higher pH values result in the precipitate of oxyhydroxides. The pH of the reaction also affects the $OH^{\bullet}$ radicals concentration; at a reduce pH values (<3) the scavenging effect of these radicals by H^{+} is severe, and for pH>3 the formation of hydroxyl radicals slows down due the hydrolysis of Fe^{3+} and the precipitation of FeOOH from the solution [54-56]. On the other hand, stability of hydrogen peroxide is also reduced at high pH values.

1.4.1.2 Effect of the catalyst load

Usually the degradation rate increases with an increase in the concentration of catalyst. However, a huge increase in the Fe^{2+} will lead to an increase in the unutilized quantity of iron salts, which will contribute to an increase in the total dissolved solids content of the effluent stream in the waste water treatment and this is not permitted [54, 55]. Moreover, at excessive Fe^{2+} concentrations scavenging parallel reactions (Eq. 10) are favored, declining process efficiency.

1.4.1.3 Effect of the initial H_2O_2 concentration

Concentration of hydrogen peroxide plays an important role on the efficiency of the degradation process. It has been observed that the percentage of degradation of a pollutant increases with an increase in the dosage of hydrogen peroxide. However, the large quantities of hydrogen peroxide act as a scavenger for the generated hydroxyl radicals (Eq. 15). Additionally, the unused portion of hydrogen peroxide contributes to chemical oxygen demand (COD), and hence an excess amount is not recommended. Moreover, the presence of

high amounts of hydrogen peroxide could affect the overall degradation efficiency of a combined Fenton-biological processes due to its harmful effects to organisms [54, 55].

1.4.1.4 Effect of initial concentration of pollutant

Generally, the lower initial concentration of the pollutant is favored to enhance the degradation of the pollutant, but the negative effects of treating large quantity of concentrated effluent needs to be analyzed before the dilution ratio is fixed [54, 55]. Even so, degradation kinetics is usually favored for higher organic loads.

1.4.1.5 Effect of the operating temperature

Reaction temperature is an important parameter but a limited number of studies are available depicting the effect of temperature on the degradation rate. Kinetics of both the radicals generation and pollutants oxidation are improved with reaction temperature. Ambient conditions can be used with a good efficiency, but it is common to find reported an optimum value for temperature around 30 °C, because above this temperature is expected that the efficient utilization of H_2O_2 decreases due to its accelerated thermal decomposition into water and oxygen [54, 56].

1.5 Photo-Fenton

Because of Fenton's reaction simplicity, it is the process most often AOP applied when it is necessary to remove recalcitrant compounds. However, it was found that irradiation of Fenton reaction with UV/Vis light strongly accelerated the degradation rate of a variety of pollutants. Photo-Fenton reaction is a combination of Fenton reagents and UV-Vis light which primarily produces hydroxyl radicals whilst photocatalytically regenerating Fe^{3+} to Fe^{2+} (Eq. 17 to Eq. 19) [20].

$$Fe^{2+} + H_2O_2 \xrightarrow{hv} Fe^{3+} + OH^- + HO^\bullet \qquad \textbf{Eq. 17}$$

$$Fe^{3+} + H_2O \xrightarrow{hv} Fe^{2+} + H^+ + HO^\bullet \qquad \textbf{Eq. 18}$$

$$H_2O_2 \xrightarrow{hv} 2HO^\bullet \qquad \textbf{Eq. 19}$$

In the photo-Fenton process the radiation not only regenerates Fe^{2+}, but also produces additional hydroxyl radicals, the species responsible for the degradation of organic compounds. As a consequence of these two effects, the photo-Fenton process is faster than the conventional (dark) Fenton process. Furthermore, since Fe^{2+} is regenerated by light with decomposition of water, rather than H_2O_2, the photo-Fenton process consumes less H_2O_2 and requires only a small quantities of iron salt [40].

The photo-Fenton process has several operation and environmental advantages. The photo-Fenton process requires only catalytic quantities of iron salt. Any residual hydrogen peroxide that is not consumed in the process will spontaneously decompose into water and molecular oxygen and is thus a "clean" reagent for itself. Thus, homogenous photo-Fenton based AOP is the leading candidate for cost efficient, environmental friendly treatment of industrial effluents on a small to moderate scale [40].

2 Aim of the thesis

The main objective of this thesis was to study some advanced oxidation processes, namely dark Fenton, direct photoloysis, photo-assisted degradation in presence of hydrogen peroxide and photo-Fenton process as a possible alternatives for the procedures tested for 5-Fu degradation. As secondary objective, we intend to develop an analytical methodology to evaluate the 5-Fu degradation degree in samples from degradation experiments.

3 Technical Description

3.1 Reagents

5-Fluorouracil, 97.7% purity (w/w) was purchased from Sigma–Aldrich (St. Louis, MO, USA). For the homogenous Fenton and in the photo-Fenton processes, iron (II) sulphate heptahydrate ($FeSO_4.7H_2O$) with 99.0% purity (w/w) was used as catalyst, hydrogen peroxide (H_2O_2) (30% v/v) as oxidant and anhydrous sodium sulphite (Na_2SO_3) (98% w/w) as quenching agent; all of them were purchased from Merck (Darmstadt, Germany). Sulphuric acid (H_2SO_4) (96% v/v) was acquired from José M. Vaz Pereira, Lda (Lisbon, Portugal), while sodium hydroxide (NaOH) (98.7% w/w) was purchased from José Manuel Gomes dos Santos, Lda (Odivelas, Portugal); both were used to adjust medium pH. Formic acid (98% v/v) (analytical grade) was from Sigma–Aldrich (St. Louis, MO, USA) and methanol (analytical grade) 99.9% purity (v/v), HiPerSolv CHROMANORM was acquired at VWR. Syringe filter with 0.2 µm PTFE membrane were purchased from VWR (Wester Chester, USA).

3.2 Standards preparation

A 5-Fluorouracil stock solution (250 mg L^{-1}) was prepared dissolving an appropriate amount of 5-Fu analytical standard in 250 mL of distilled water. Working solutions of 5-Fu were prepared by dilution of the previous stock solution in water and were kept in the refrigerator until their use.

3.3 Analytical procedures

3.3.1 Analysis by HPLC-DAD

The 5-Fu degradation was followed by high performance liquid chromatography with diode array detection (HPLC-DAD). The HPLC-DAD consists in an L-2130 pump, an L-2200 auto-sampler and an L-2455 diode array detector (DAD). The chromatographic separation was achieved by a Purospher® Star RP-C18 endcapped column (250 mm × 4 mm, 5 µm) using a mobile phase composed of 97% (v/v) distilled water and 3% (v/v) of methanol, both acidified with 0.01% of formic acid (v/v), at isocratic conditions, with a flow rate of 0.2 mL min^{-1}. Injection volume was 20 µL. The spectra acquisition was done from 220 to 400 nm and 5-Fu was quantified at 266 nm, with a retention time of 23.80 ± 0.02 min. The analytical method was optimized based on the methods described in the literature.

3.3.2 Total organic carbon (TOC)

Mineralization was monitored through total organic carbon (TOC) losses by measuring the total (TC) and the inorganic carbon (IC) in a Shimadzu 5000A analyzer according to the standard method 5310 D of the American Public Health Association [57]. TOC of each sample was calculated by subtracting the IC to the TC. The reported values of TOC represent the average of at least two measurements; in most cases each sample was injected three times or more, validation being performed by the apparatus only if the coefficient of variation (CV) is smaller than 2%.

3.3.3 Toxicity

The toxicity assays were carried out in a Microtox Model 500 Analyzer by measuring the inhibition of *Vibrio fischeri* bioluminescence according to the standard DIN/EN/ISO 11348-3 [58]. The toxicity of the initial solution and of the generated effluents was determined based on the changes in *V. fischeri* luminescence after 5, 15 and 30 min of exposure.

3.4 Fenton oxidation

In this study, the condition in which the Fenton process has its optimum performance was determined using classical pre-tests, varying one parameter at a time while keeping the others constant. The studied parameters were the temperature and the concentrations of hydrogen peroxide, Fe (II) and 5-Fu. The Fenton process was carried out in a 250 mL batch jacketed reactor filled with 200 mL of a 5-Fu aqueous solution. The temperature was kept constant using a Huber thermostatic bath (Polystat CC1 unit). After the temperature stabilization, the solution pH was adjusted to the optimum level (pH 3) using H_2SO_4 (1 M). To measure the solution temperature and pH, a thermocouple and a pH electrode (WTW, SenTix 41 model), connected to a pH-meter from WTF (model Inolab pH Level 2) were used. Afterward, a predetermined amount of $FeSO_4.7H_2O$ was added, the solution being always stirred using a magnetic stirrer. When $FeSO_4.7H_2O$ was dissolved H_2O_2 was added, this representing the initial instant (t=0) of the experiments. Samples were periodically taken from the reactor along the reaction. In order to eliminate the residual H_2O_2 for HPLC analysis, the pH was adjusted to 9±1 with NaOH (0.1 mol L^{-1}). The remaining H_2O_2 in samples for TOC analysis was eliminated by adding an excess of Na_2SO_3. All samples were filtered with 0.2 μm PTFE syringe filters and stored at −20 °C until further analysis.

3.5 Radiation-assisted processes

The degradation of 5-Fu in the presence of radiation was investigated exposing 250 ml of a 5-Fu aqueous solution (50 mg L^{-1}) to a 150 W medium-pressure mercury vapor lamp (Heraeus TQ 150), whose more intense line is at 366 nm. The lamp emission spectrum is presented in **Figure 3.1**. A quartz jacket with water recirculation was employed to cool the irradiation source and cancel the infrared radiation, thus preventing any heating of the solution. Depending on the kind of experiment performed, the degradation reaction was left to occur only in the presence of radiation (direct photolysis), in the presence of radiation and hydrogen peroxide or combining radiation with Fenton reagents (photo-Fenton process). Samples were periodically taken from the reactor. As indicated in section 3.4, different procedures were adopted to quench the reactions: pH adjustment in samples intended for HPLC analysis and addition of an excess of Na_2SO_3 for TOC monitoring. All samples were filtered with 0.2 μm PTFE syringe filters and stored at −20 °C until further analysis.

In order to cut-off the UV-B and UV-C emission lines in direct photolysis experiments, a DURAN 50® glass cooling jacket was placed around the UV-Vis lamp, resulting emission lines at λ_{exc} = 365, 405, 436, 646 and 678 nm.

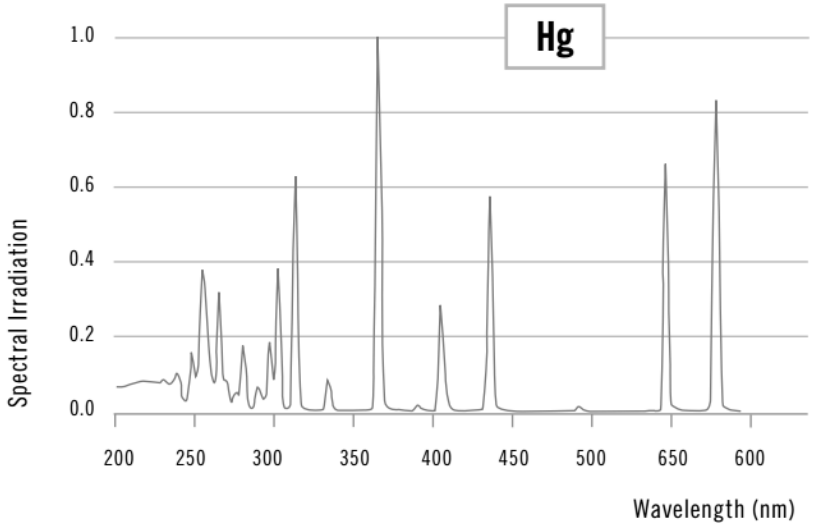


***Figure 3.1** Emission spectrum of the Heraeus TQ-150 UV-Vis lamp (manufacture data).*

4 Results and Discussion

4.1 Development and validation of the HPLC-DAD method for 5-Fu quantification in waters

The aim of this work was to study the performance of different degradation processes regarding the treatment of waters contaminated with 5-Fu. For that, it was necessary to develop an analytical methodology to evaluate the 5-Fu degradation degree in samples from degradation experiments. The method was developed taken into account the necessity of achieving reliable 5-Fu analytical responses under alkaline conditions (required to stop the reaction, as described in the previous chapter) and in the presence of Fenton's species (iron (II) and hydrogen peroxide). On the other hand, an effort was done in order to increase as much as possible the retention time of 5-Fu, given the future interest of detecting peaks of the degradation by-products at lower retention times. The analytical methodology was finally validated determining the linearity range, the limits of detection (LOD) and quantification (LOQ) and evaluating the precision and accuracy.

4.1.1 Analytical method by direct injection in the HPLC-DAD

Concerning the method development, different analytical conditions (composition and acidity of the mobile phase, temperature and flow rate) were tested and their effects on the 5-Fu analytical response were assessed. The main objective was to find the conditions under which the retention time of 5-Fu peak was the maximum, without compromising the analytical response (peak resolution and area), as well as the excessive time of analysis. Typically, the degradation products have lower retention times compared to the parent compound and for that reason, an increase on the 5-Fu retention time would allow to obtain a larger window for degradation by-products detection.

The best performance was obtained at 25 °C, in isocratic conditions (0.2 mL min^{-1}) and with the following mobile phase: 97% water acidified with 0.01% of formic acid (v/v) and 3% methanol acidified with 0.01% of formic acid (v/v). Under these conditions, the retention time of 5-Fu is 23.80±0.02 min. More details are given in Appendix B - Table B 1.

A chromatogram of a 5-Fu aqueous solution (50 mg L^{-1}) is presented in Figure 4.1.

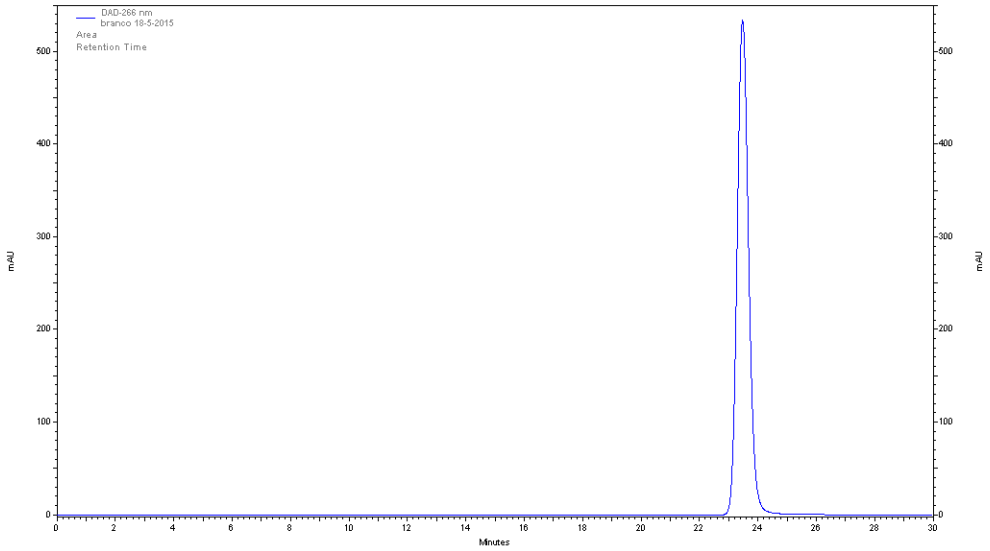


Figure 4.1 *Chromatogram of a 5-Fu aqueous solution (50 mg L^{-1}). Analytical conditions: isocratic mode (0.2 ml min^{-1}); (97% water acidified with 0.01% of formic acid (v/v) and 3% methanol acidified with 0.01% of formic acid (v/v); 25 ºC; 20 µL of volume of injection.*

4.1.2 Validation of the method

The purpose of method validation is to ensure that the analytical method is accurate, specific, reproducible and robust over the specified range under which an analyte will be analyzed. In this work the evaluated parameters to ensure the acceptability of the analytical method were linearity, accuracy and precision.

4.1.2.1 Linearity range and limits of detection and quantification

The linearity is the ability of an analytical procedure to provide results that are directly proportional to the concentration of the analyte in the samples. Additionally, the linearity range is the interval between the upper and lower levels of analyte (inclusive) that have been demonstrated to be determined with precision, accuracy and linearity [59].

Under the above described experimental conditions, a linear relationship was observed by plotting 5-Fu concentration against peak area, Figure 4.2. The corresponding concentration ranged from 0.02 to 50 mg L^{-1} of 5-Fu. The slope, intercept and correlation coefficient obtained by the linear least squares regression treatment are presented in Table 4.1, along with the standard deviation of residuals ($s_{y/x}$), of intercept (s_a), and of slope (s_b). $S_{y/x}$ is a measure of the extent of deviation of the found (measured) area-values from the calculated ones by the calibration curve equation.

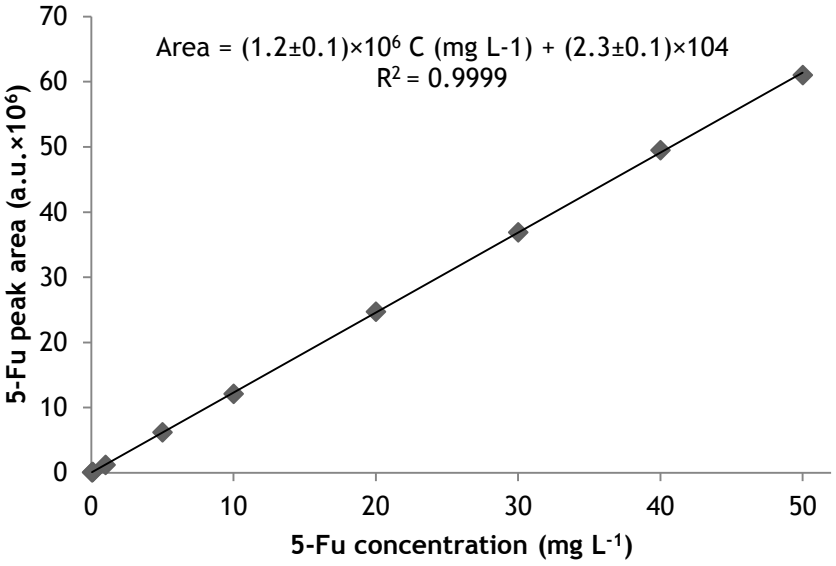


Figure 4.2 *Calibration curve for 5-Fu quantification in water by HPLC-DAD.*

Limit of detection (LOD) and limit of quantification (LOQ) were determined by the signal-to-noise ratio of 3 and 10, respectively. LOD is defined as the lowest concentration of analyte in the sample that can be detected. Limit of quantification is defined as the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy. LOD and LOQ were calculated and are presented in Table 4.1.

Table 4.1 *Regression and statistical parameters for the determination of 5-Fu in waters by HPLC-DAD*

Linearity range (mg L^{-1})	Regression data			$S_{y/x}$ [d]	S_a [e]	S_b [e]	LOD [g] (mg L^{-1})	LOQ [h] (mg L^{-1})
	a [a]	b [b]	R [c]					
0.02-50	2.3×10^4	1.2×10^6	0.999	0.2×10^6	0.1×10^6	0.1×10^4	0.006	0.02

[a] Intercept; [b] Slope; [c] Correlation coefficient; [d] Standard deviation of residuals; [e] Standard deviation of intercept; [f] Standard deviation of slope; [g] Limit of detection, [h] Limit of quantification.

The quality control laboratories typically use three different criteria for admitting that a method is suitable for use in analysis [60]: a) relative standard deviation of the slope (s_a/a) should be lesser than 5%; b) interception should contains the origin ($b\text{-}s_a < 0 < b + s_a$); c) the correlation coefficient of the calibration curve should be greater than 0.995. Therefore, it can be concluded that the calibration curve is suitable for the purpose of this analysis since it meets all the criteria specified above: the relative standard deviation of the slope was 0.34%, the correlation coefficient of the calibration curve was 0.999, and the confidence limits for the interception contain the origin.

4.1.2.2 Precision

The precision expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same sample under the prescribed conditions. Precision is expressed as the percent relative standard deviation (RSD%). In this work precision was evaluated by repeatability and intermediate precision [59]. The repeatability was evaluated by injection of three 5-Fu standard solutions (0.1, 10 and 50 mg L^{-1}) six times in the same day and under the same conditions (intra-day test). The intermediate precision was evaluated by injection of the same standard solutions in 6 different days (inter-day). The results of precision (expressed as relative standard deviation) are presented in Table 4.2.

***Table 4.2** Repeatability and intermediate precision for the determination of 5-Fu in waters by HPLC-DAD*

	5-Fu concentration (mg L^{-1})		
n = 6	**0.1**	**10**	**50**
Repeatability (RSD%)	2.15	0.19	0.14
Intermediate Precision (RSD%)	4.02	1.40	1.06

The results showed that there are higher variations in the response for lower 5-Fu concentrations than for higher ones, but values well below 10% were achieved for all range of concentrations, which are very good.

4.1.2.3 Accuracy

Accuracy is the closeness of the results obtained by the analytical method to the true value [59]. Accuracy was assessed by calculating the percent recovery of the analyte at three different concentrations (0.1, 10 and 50 mg L^{-1}) at five different scenarios.

The first test intended to evaluate if the material of the syringe filter retains 5-Fu since this step is a required procedure before the HPLC analysis. For that, three aqueous 5-Fu standards (0.1, 10 and 50 mg L^{-1}) were filtered with a 0.2 µm PTFE membrane and the analytical responses obtained for them were compared to the corresponding standards prepared with filtered distilled water.

The second test was performed in 5-Fu solution at pH 10. This test intended to evaluate the effect of high pH (required for stop the Fenton reaction, as described in section3.4) in the 5-Fu stability. The effect of pH was assessed by adjusting the solution at pH 3 with H_2SO_4 and then adding a predetermined volume of NaOH (1 M) to raise the pH to 10. The analytical

response was compared with the one obtained for the same standard prepared in distilled water without pH adjustment.

In the third test the effect of Fe^{2+} (the catalyst in the Fenton reaction) was evaluated. The test was performed with a concentration of Fe^{2+} of 5×10^{-4} M, which is the highest concentration used in Fenton's reactions performed in this work. The effect of Fe^{2+} was studied by adjusting the solution pH at pH 3 (with H_2SO_4), adding $FeSO_4$ and finally, raising the pH to 10 (with NaOH).

The effect of H_2O_2 was assessed by evaluation the 5-Fu analytical response in the presence of $2.4x10^{-1}$ M of H_2O_2, the highest concentration used herein for Fenton's reactions. The procedure adopted was the same described previously for Fe^{2+}. The only difference deals with the addition of H_2O_2 instead of Fe^{2+}.

The synergistic effect of Fenton's reagents was also evaluated. The solution pH was adjusted at 3 (with H_2SO_4) and then a predetermined mass of $FeSO_4$ was added (5×10^{-4} M), followed by pH adjustment to 10 (with NaOH). Afterwards 2.4×10^{-1} M of H_2O_2 was added. It is worth noting that H_2O2 was added at the end to avoid Fenton reaction and inherently 5-Fu degradation; H_2O_2 decomposes at alkaline conditions

The recovery values obtained for all referred test conditions are indicated in Table 4.3.

Table 4.3 *Evaluation of the accuracy of the HPLC-DAD method for the determination of 5-Fu in waters*

Recovery (n = 3)	5-Fu concentration (mg L^{-1})		
	0.1	**10**	**50**
Stability of 5-Fu after filtration	97 ± 3	102 ± 2	100.7 ± 0.7
Stability of 5-Fu at pH 10	101 ± 3	98 ± 2	98 ± 2
Stability of 5-Fu in solution with $FeSO_4$	95 ± 4	92 ± 8	98 ± 2
Stability of 5-Fu in solution with H_2O_2	89 ± 10	77 ± 23	87 ± 13
Stability of 5-Fu with Fenton's reagents	101.1 ± 0.1	98 ± 2	85 ± 15

The good recovery results enable a reliable quantification of 5-Fu in the tested conditions, even at the lowest contamination level assessed.

4.2 Fenton oxidation

Fenton's oxidation has been extensively studied for the degradation of a large number of recalcitrant compounds. Herein, it's performance was evaluated for the treatment of waters contaminated with 5-Fu. A parametric study was done regarding the assessment of the effect of some parameters in the 5-Fu degradation process: temperature, the amount of catalyst

(Fe^{2+}), the dose of hydrogen peroxide (H_2O_2) and the initial concentration of 5-Fu. The reaction pH was fixed at 3.0, the optimum value commonly reported for Fenton's oxidation of many organics [e. g., 56-58, 64].

4.2.1 Parametric study of the variables affecting the Fenton's reaction

4.2.1.1 Effect of the temperature

Temperature plays an important role in the degradation of pollutants in the Fenton's process because it affects the rate of reaction between H_2O_2 and Fe^{2+} as well as the rate of hydroxyl radicals attack to the organics. The effect of temperature in the 5-Fu degradation by Fenton's reagent was studied at three different temperatures: 15, 30 and 45 °C. As expected and predicted by Arrhenius law, an increase of the temperature led to an increase of the 5-Fu degradation rate, Figure 4.3 [53].

At higher temperatures the rate of reaction between H_2O_2 and Fe^{2+} increases leading to the faster formation of hydroxyl radicals, which in turn results in enhanced degradation efficiency. However, similar 5-Fu degradation degrees were observed after 60 min for experiments performed at 30 and 45 °C. These results, along with the lower operating costs observed at lower temperatures (lower energy consumption), led to the selection of the temperature of 30 °C as the most cost-effective one (within the temperatures studied). Besides, it is common to find reported on the literature an optimum value for temperature of around 30 °C [53, 61].

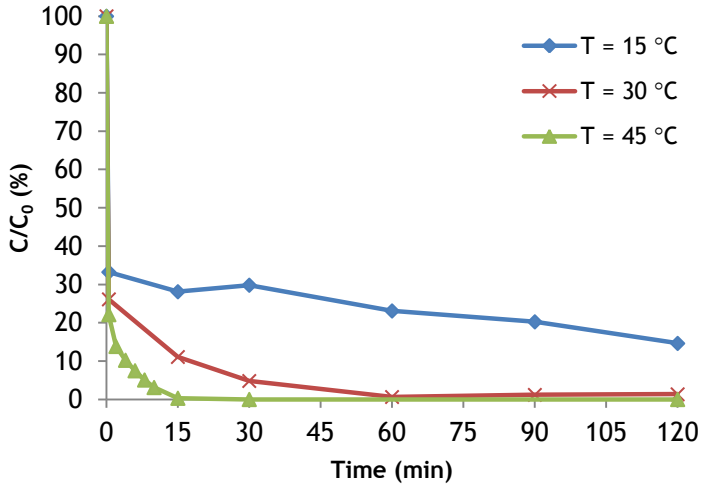


Figure 4.3 *Temperature effect on the 5-Fu degradation in water ($[H_2O_2] = 6.5 \times 10^{-3}$ M, $[Fe^{2+}] = 5.0 \times 10^{-4}$ M, $pH_0 = 3$ and $[5\text{-Fu}]_0 = 3.8 \times 10^{-4}$ M).*

4.2.1.2 Effect of iron salt concentration

The loading of ferrous ion is also an important parameter in the efficiency of Fenton process. The optimization of Fe^{2+} is necessary as an overdose of iron would product to many ferrous ions, which would scavenge hydroxyl radicals resulting in a decrease of degradation efficiency. Also the treatment costs shall be increasing by an excess amount of iron salts; moreover, the amount of sludge increases leading to enhanced treatment costs [61]. Figure 4.4 shows the effect of Fe^{2+} dose on the degradation of 5-Fu.The results reveal that the 5-Fu degradation increases as the dose of catalyst increases.

Moreover, for the catalyst concentrations tested, it is observed a rapid decrease in 5-Fu concentration followed by a more gradual decline, which can be explained by the inter-conversion of Fe^{2+}/Fe^{3+}. In the Fenton reaction, Fe^{2+} is quickly converted to Fe^{3+} and 5-Fu was degraded rapidly. However, Fe^{3+} is converted to Fe^{2+} more slowly, resulting in the decrease of 5-Fu degradation rate, as also reported elsewhere for phenol degradation [62].

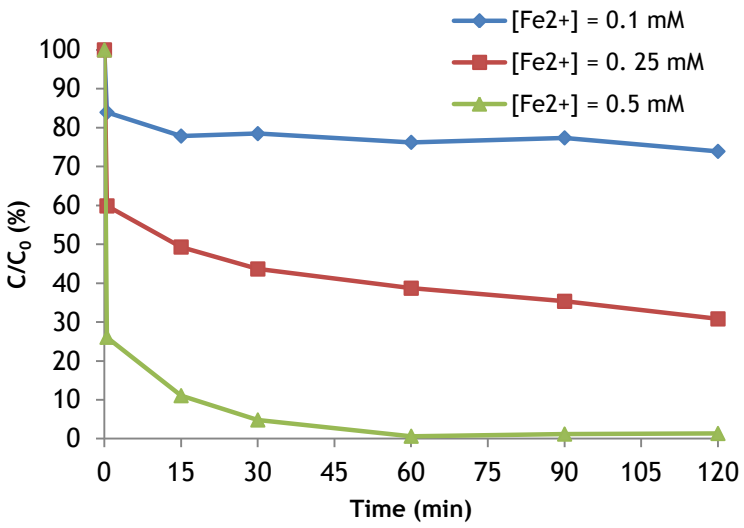


Figure 4.4 Fe^{2+} concentration effect on the 5-Fu degradation (T = 30 °C, $[H_2O_2] = 6.5\times10^{-3}$ M, $pH_0 = 3$ and $[5\text{-Fu}]_0 = 3.8\times10^{-4}$ M).

4.2.1.3 Effect of the H_2O_2 concentration

Hydrogen peroxide is a strong oxidizing agent. The loading of hydrogen peroxide plays a crucial role in the efficiency of the degradation process and the main costs associated with the Fenton process are often due to H_2O_2 [61]. Thus, the loading of hydrogen peroxide should be adjusted in order to maximize the efficiency of its use. The effect of the H_2O_2 dose on 5-Fu degradation process was investigated over a range of 0.96 to 240 mM corresponding to H_2O_2 to 5-Fu molar ratio of 2.5:1 and 625:1 (Figure 4.5). The results showed that the degradation of 5-Fu is very quick for all H_2O_2 concentrations studied and only slightly

differences were observed in the first 15 minutes, being the process slower for lower doses of oxidant.

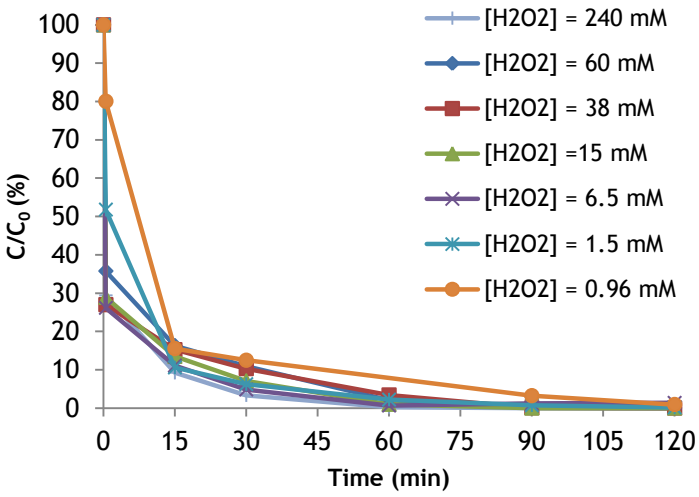


***Figure 4.5** Effect of H_2O_2 concentration on the 5-Fu degradation (T = 30.0 °C, $[Fe^{2+}] = 5.0 \times 10^{-4}$ M, $pH_0 = 3$ and $[5\text{-Fu}]_0 = 3.8 \times 10^{-4}$ M).*

To obtain further insight into the effect of H_2O_2 concentration on 5-Fu degradation, the 5-Fu mineralization was investigated by assessing TOC removal. TOC removal indicates the extent of the mineralization (rather than just transformation) of the target compound. Figure 4.6 presents the effect of the hydrogen peroxide dose (H_2O_2 to Fe^{2+} molar ratios of 2:1, 120:1 and 500:1) on 5-Fu mineralization. It can be seen the best TOC removal of approximately 25% was reached at the higher oxidant dose of 240 mM (molar ratio of 500:1) after 120 min of reaction. The TOC removal efficiency increases with the increase of H_2O_2 concentration. At high concentrations of H_2O_2 occurs the increase of the production of hydroxyl radicals that are responsible for 5-Fu degradation. Since hydroxyl radicals are also able to degrade by-products, the increased amount of hydroxyl radicals will lead to higher percentage of mineralization. The results showed that although 5-Fu was completely removed after 120 min at different H_2O_2 loadings, mineralization is far from being good, suggesting the refractory nature of the oxidation by-products towards hydroxyl radicals attack.

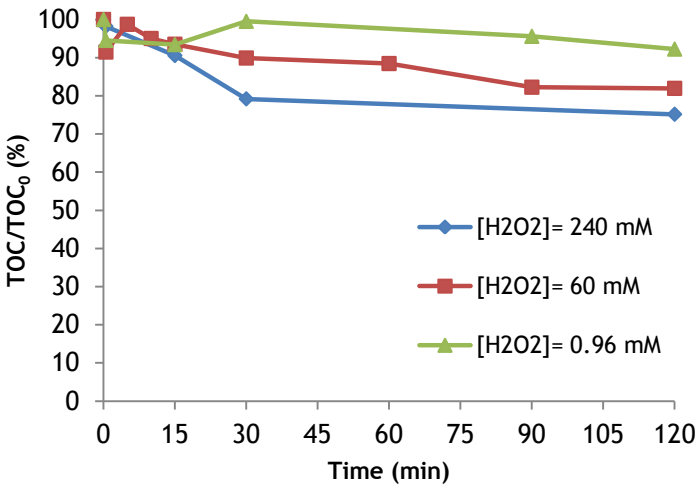


Figure 4.6 *Effect of the H_2O_2 concentration on 5-Fu mineralization (T = 30.0 °C, $[Fe^{2+}] = 5.0 \times 10^{-4}$ M, $pH_0 = 3$ and $[5\text{-Fu}]_0 = 3.8 \times 10^{-4}$ M).*

In order to evaluate the effect of reaction time in the 5-Fu mineralization, the reaction with the highest dose of oxidant was performed during 24 hours. The results are presented in Figure 4.7 and Figure 4.8. The results showed that, although 5-Fu could be completely removed after 60 min with a molecular ratio of 500:1 (H_2O_2:Fe^{2+}) by Fenton reaction, only about 50% of mineralization occurs after 24 hours of the reaction.

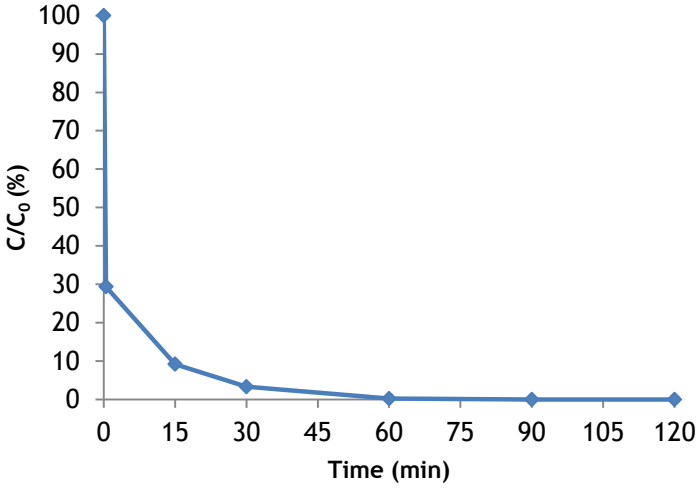


Figure 4.7 *5-Fu degradation by Fenton process (T = 30.0 °C, $[H_2O_2] = 2.40 \times 10^{-1}$ M, $[Fe^{2+}] = 5.0 \times 10^{-4}$ M, $pH_0 = 3$ and $[5\text{-Fu}]_0 = 3.8 \times 10^{-4}$ M).*

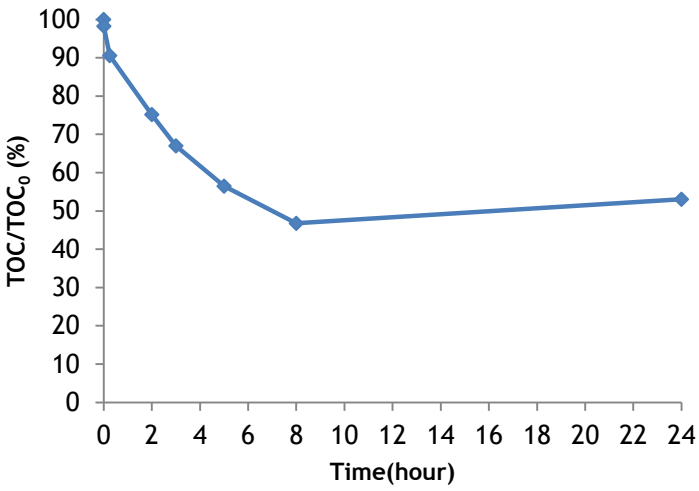


Figure 4.8 *5-Fu mineralization by Fenton process (T = 30.0 °C, $[H_2O_2]$ = 2.40 × 10^{-1} M, $[Fe^{2+}]$ = 5.0 × 10^{-4} M, pH_0 = 3 and $[5\text{-Fu}]_0$ = 3.8 × 10^{-4} M).*

4.2.1.4 Effect of initial 5-Fu concentration

The parametric study of the variables affecting the Fenton's reaction was up to now done with a 5-Fu concentration of 50 mg L^{-1}. However, as 5-Fu has been detected at lower concentrations in the environment, the best operating conditions obtained for a concentration of 5-Fu of 50 mg L^{-1} were tested at 5 mg $L^{-1}$and 0.5 mg L^{-1}, in order to evaluate if the favorable operating conditions found can be applied for lower initial concentrations of 5-Fu. However, when decreasing the cytostatic initial concentration, the dose of oxidant and catalyst were decreased in the same proportion, to keep constant in all experiments the ratio 5-Fu:H_2O_2:Fe^{2+}. The results presented in Figure 4.9 indicated that the best performance is obtained for the highest initial concentrations because the reaction kinetics is accelerated.

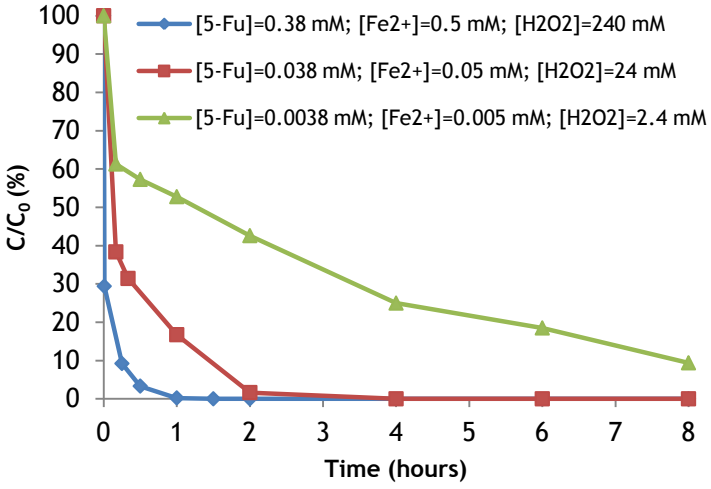


Figure 4.9 *Effect of 5-Fu initial concentration on the 5-Fu degradation in water (T = 30.0 °C, pH_0 = 3, molar ratio of 500 (Fe^{2+}:5-Fu) and 625 (H_2O_2:5-Fu).*

Fenton processes reveals to be an effective method for 5-Fu degradation. Nevertheless, the following drawbacks have been described in the literature: rather high costs and risks due to the storage and transportation of H_2O_2; need of important amounts of chemicals for acidifying effluents at pH 3; accumulation of iron sludge that must be removed in the end of the treatment; impossibility of overall mineralization due to the formation of Fe(III)-carboxylic acid complexes, which cannot be efficiently destroyed with bulk $HO^{\bullet}$ [63]. Thus, in order to overcome some of these drawbacks, Fenton process has been coupled to various processes allowing a better degradation of organic pollutants. One possibility is to use photo-assisted technologies, which are studied in the following section.

4.3 Photo-assisted technologies

The photochemical technologies present some advantages as the facts of being simple, clean and generally more efficient than chemical AOPs. Consequently, use of radiation has been coupled with powerful oxidants such as H_2O_2, including, in some cases, a catalysis, resulting in various kinds of important photochemical AOPs. These processes are able to degrade and/or destroy pollutants by means of several possible reactions, including direct photodecomposition, based on irradiation, excitation and degradation of pollutant molecules, oxidation by direct action of H_2O_2, and oxidation by photocatalysis inducing the formation of $HO^{\bullet}$ radicals.

In this work direct photolysis and two photochemical technologies, photodegradation with H_2O_2, and photo-Fenton, were studied in order to evaluate their performance in the degradation of 5-Fu.

4.3.1 Photolysis

In the photolytic degradation, the transformation of a reactant molecule is achieved by the absorption of radiant light. The reactant molecule absorbs the light energy and is transformed into other chemicals forms by the hemolytic cleavage of active molecules to form the degradation products [63, 64]. The reported results in the literature have shown that the efficiency of photolysis on 5-Fu degradation can depend of different factors such as its light absorption (that is affected by the pH of the solution) and the wavelength of the radiation source [14, 48].

4.3.1.1 Effect of different irradiation wavelengths

In order to evaluate the effect of different irradiation wavelengths, direct photolysis of 5-Fu was carried out at two different irradiation conditions: polychromatic light in the range 200–600 nm (UV-Vis) and polychromatic light at λ ≥ 365 nm (mostly visible light irradiation, Vis). A quartz jacket was used in the experiments coupled with a polychromatic light in the range 200–600 nm (UV-Vis), because quartz transmits all the radiation emitted by the mercury lamp, (Figure 4.10). In order to cut-off the UV light, a glass jacket (Duran 50) was used because glass does not transmit light with the wavelength shorter than 350 nm (Figure 4.10).

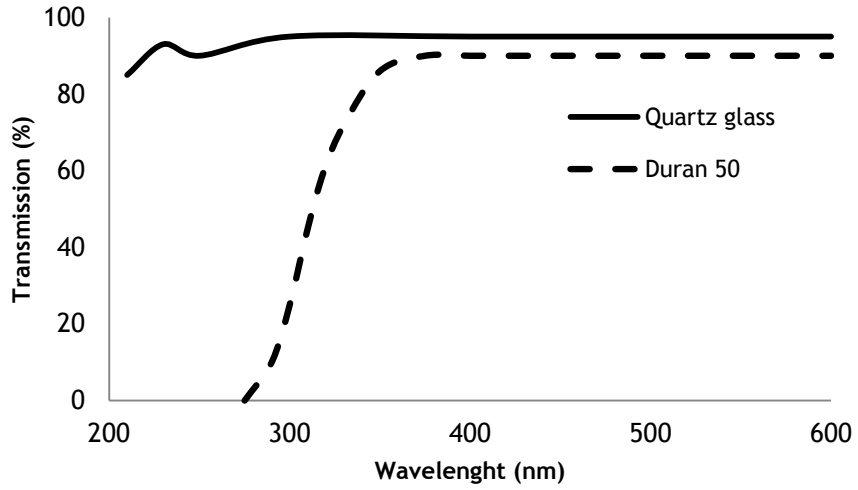


Figure 4.10 *Transmission of quartz glass and Duran 50 as a function of wavelength (data provided by the manufacturer).*

The results presented in Figure 4.11 indicate that when the radiation source emits light with wavelengths between 200 and 600 nm, 5-Fu is completely degraded after 6 hours of irradiation by direct photolysis. However, when UV radiation is removed, the degradation rate of 5-Fu decreases.

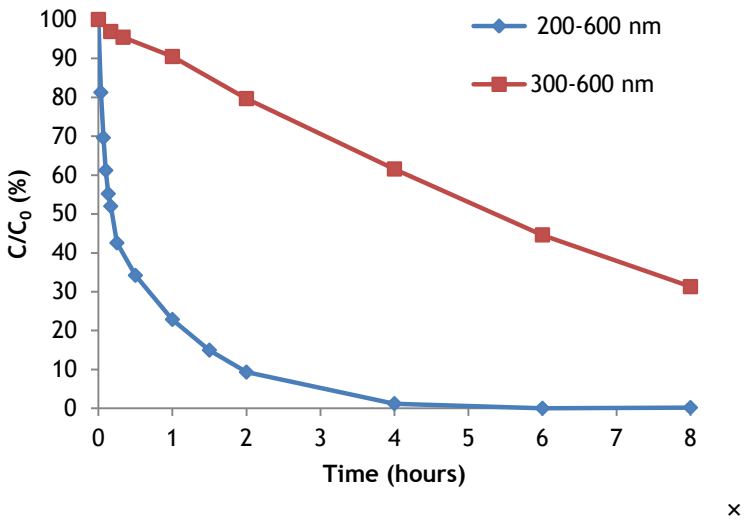


Figure 4.11 *Effect of different irradiation wavelengths on the 5-Fu degradation in water by direct photolysis (pH = 3; T = 30 °C; $[5\text{-Fu}]_0 = 3.8 \times 10^{-4}$ M).*

These results can be explained by 5-Fu's absorbance spectrum (Figure 4.12). 5-Fu exhibit a maximum absorbance at 266 nm, therefore, since glass does not transmit light with the wavelength shorter than 350 nm, as expected, 5-Fu's degradation rate decreases due to the decrease in the 5-Fu absorption.

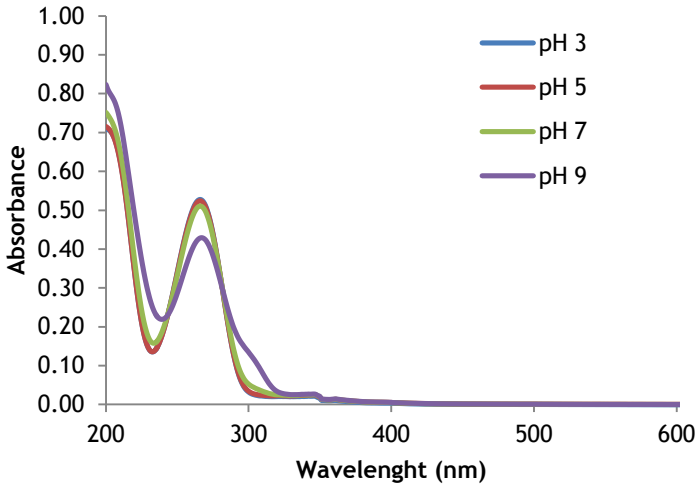


Figure 4.12 *Effect of pH on absorbance spectra of 5-Fu.*

4.3.1.2 Effect of the pH of the solution

Several studies have shown that the pH of the solution influences the reactivity of the chemical compounds, increasing or decreasing the rate of the reaction [65]. In this work the pH effect was evaluated at three different pH values: 3, 7 and 9. The results presented in Figure 4.13 indicate that 5-Fu degradation rate increases for higher pH values. This may be

explained by the increase of absorbance at longer wavelengths to higher pH values. At pH > 7, 5-Fu has a maximum absorbance peak at 266 nm, but additionally it absorbs radiation in the range of wavelengths 280 to 320 nm (Figure 4.12). This feature is associated with the formation of the deprotonated form of 5-Fu [14]. Similar results are obtained at pH 7 and 9, however it is possible to verify that the degradation is fastest at pH 7. According to Lin et al [48] the rate of reaction should increase with increasing pH; however, the results obtained may be related to the temperature variation (T = 30 ± 1 °C) since the experiments were performed on different days.

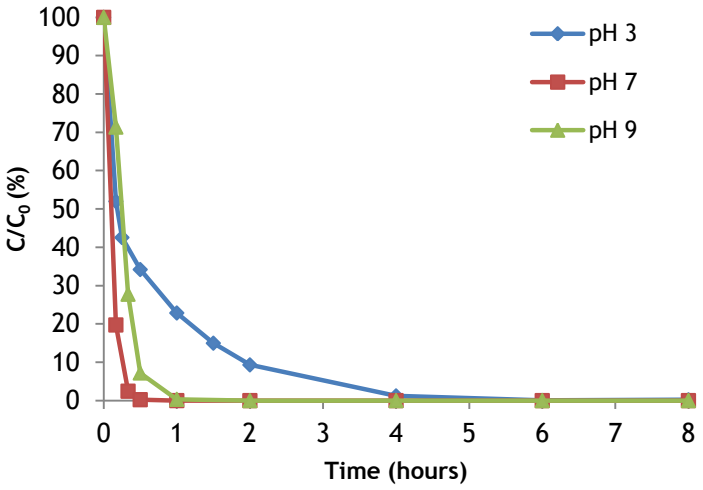


Figure 4.13 *Effect of the pH of the solution on the 5-Fu degradation in water by direct photolysis (wavelength 200 to 600 nm; T = 30 °C; $[5\text{-Fu}]_0 = 3.8 \times 10^{-4}$ M).*

4.3.2 Photodegradation with H_2O_2

The mechanisms of photodegradation with H_2O_2 have been investigated extensively, and it has been found that the rate of degradation of an organic compound using radiation with H_2O_2 results from the contribution of two pathways: direct photolysis and hydroxyl radical attack [66]. Direct attack by the oxidant itself should not be excluded, but in this case its contribution is negligible.

The degradation of 5-Fu by photo-assisted degradation with H_2O_2 was investigated in the presence of different initial concentrations of H_2O_2. Similar results were obtained for a H_2O_2 concentration of 0.96 mM and 240 mM (Figure 4.14). The reason for such phenomenon is that in the presence of high concentration of H_2O_2, more hydroxyl radicals were generated, but the scavenging effect of H_2O_2 became significant. On the one hand, hydroxyl radicals reacted with the excess H_2O_2 to produce hydroperoxyl radicals (Eq. 21), which consume part of hydroxyl radicals. On the other hand, the hydroperoxyl radicals produced as a result of Eq. 21

could also react with hydroxyl radicals (Eq. 22). Furthermore, hydroxyl radicals produced at high concentration, dimerize to H_2O_2 (Eq. 23) [63].

$$H_2O_2 + hv \rightarrow 2HO^{\bullet} \quad \text{Eq. 20}$$

$$H_2O_2 + HO^{\bullet} \rightarrow HO_2^{\bullet} + H_2O \quad \text{Eq. 21}$$

$$HO_2^{\bullet} + HO^{\bullet} \rightarrow +H_2O + O_2 \quad \text{Eq. 22}$$

$$HO^{\bullet} + HO^{\bullet} \rightarrow H_2O_2 \quad \text{Eq. 23}$$

The results showed that the degradation of 5-Fu is very quick for both H_2O_2 concentrations studied and only slightly differences were observed in the first 6 minutes, the process being slower for lower dose of oxidant.

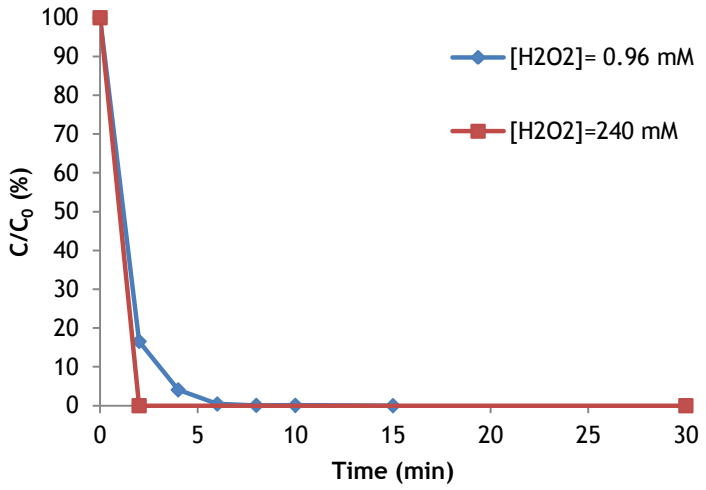


Figure 4.14 *Effect of H_2O_2 concentration on the 5-Fu degradation in waters by photodegradation with H_2O_2 (wavelength 200 to 600 nm; T = 30 °C; $[5\text{-Fu}]_0 = 3.8 \times 10^{-4}$ M, pH = 3).*

4.3.3 Photo-Fenton

Fenton reaction can be photo-assisted by using UV/Vis radiation to stimulate the catalytic reduction, in H_2O_2 aqueous solution, of Fe^{3+} into Fe^{2+}, which increases the formation of hydroxyl radicals. In this work the efficiency of photo-Fenton process was evaluated using two different operating conditions, previous tested for Fenton reaction. The results are presented in Figure 4.15. The results showed that the best 5-Fu degradation was achieved at the highest H_2O_2 concentration, being 5-Fu degradation extremely fast. These results may be explained by the fact that at highest initial concentration of H_2O_2 was generated a higher quantity of hydroxyl radicals leading to faster 5-Fu degradation.

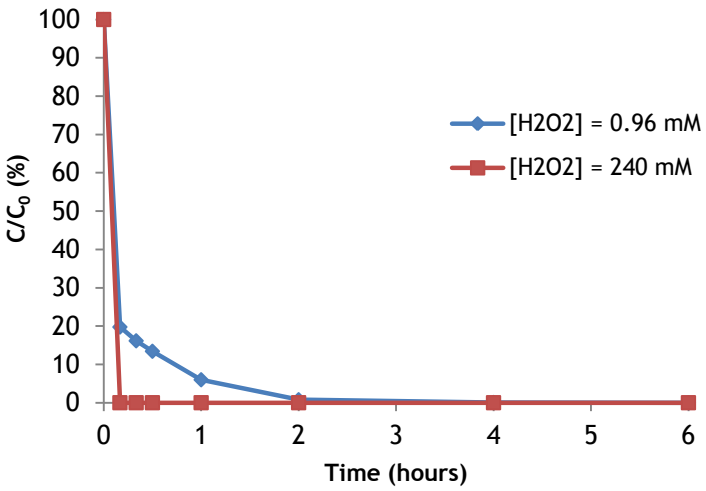


Figure 4.15 *5-Fu degradation by photo-Fenton process (wavelength 200 to 600 nm; T = 30 °; $[5\text{-Fu}]_0 = 3.8 \times 10^{-4}$ M, pH = 3, $[Fe^{2+}]_0 = 5.0 \times 10^{-4}$ M).*

4.4 Processes comparison

Based on the 5-Fu degradation one can say that all of the studied methods can be used for removal of 5-Fu in waters. In terms of the degradation rate and considering only the primary elimination of the parent compound which was monitored by HPLC-DAD, photo-Fenton and photodegradation with hydrogen peroxide were the fastest methods followed by dark Fenton process and direct photolysis (Figure 4.16). The results may be explained by the contribution of two pathways: direct photolysis and hydroxyl radical attack in both processes.

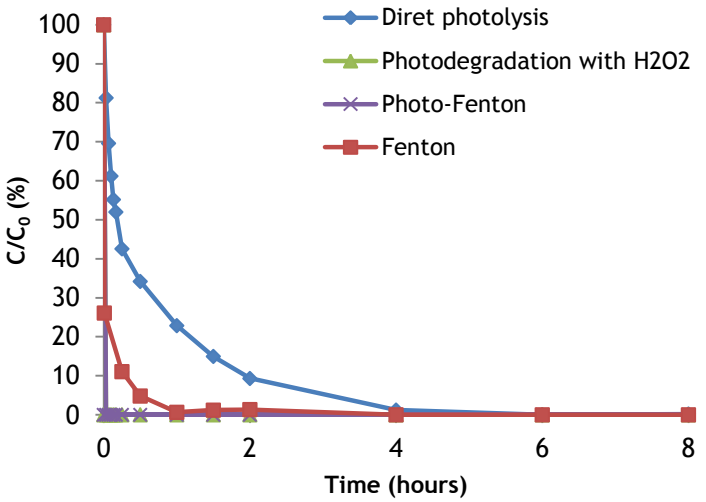


Figure 4.16 *5-Fu degradation in waters by Fenton oxidation, direct photolysis, photodegradation with H_2O_2 and photo-Fenton process (T = 30 °C; $[5\text{-Fu}]_0 = 3.8 \times 10^{-4}$ M, pH = 3, $[Fe^{2+}]_0 = 5.0 \times 10^{-4}$ M, $[H_2O_2] = 2.40 \times 10^{-1}$ M, wavelength 200 - 600 nm).*

Although all studied process were effective in degrading 5-Fu, just photodegradation with hydrogen peroxide and photo-Fenton allowed to obtain the complete mineralization of 5-Fu. The results are presented in Table 4.4. The best mineralization obtained for photo-Fenton process may be explained by the faster generation of hydroxyl radicals due to the catalytic reduction, in H_2O_2 aqueous solution, of Fe^{3+} into Fe^{2+} assisted by radiation.

Table 4.4 5-Fu mineralization by Fenton, direct photolysis, photodegradation with H_2O_2 and photo-Fenton processes

	$[Fe^{2+}]$ mM	$[H_2O_2]$ mM	$t_{5\text{-Fu degradation}}$	Mineralization (%)
Fenton oxidation	0.5	240	60 min	50%*[a]
Direct photolysis	-	-	4 h	73%*[a]
Photodegradation with H_2O_2	-	240	2 min	100%*[a]
Photo-Fenton	0.5	240	2 min	100%*[b]

*These results are indicative, it is necessary to perform more tests to confirm the results; [a] mineralization after 8 hours; [b] mineralization after 15 minutes.

4.4.1 Degradation products

The appearance of new peaks in the chromatograms of the elution profile of 5-Fu during its degradation processes is an indicative of the formation of transformation products, also called by-products. As mostly very little is known about toxicological properties of the by-products generated during the treatment processes, the identification of the most relevant by-products is an important issue, because they can be recalcitrant, and even more toxic than the parent compound.

The generation of transformation products can be divided in two different steps, firstly the transformation products are formed directly from the degradation of 5-Fu. Then, the primary transformation products are further degraded and form the secondary degradation products.

Although HPLC-DAD is not the most appropriate method for by-product analysis, this analytical method allows having an insight into some of the products forming during the reaction. For the identification of the degradation products other analytical method would be necessary, such as LC-MS. In the next subsection will be presented the 5-Fu elution profiles obtained by HPLC-DAD for the degradation processes studied.

In the following section are presented the chromatograms obtained from the injection of different samples collected along the degradation processes in the best operation conditions. The operating conditions are presented in Table 4.5.

Table 4.5 Operating conditions of 5-Fu degradation processes

Process	$[Fe^{2+}]$ (M)	$[H_2O_2]$ (M)	pH_0	Temperature (°C)	Radiation
Fenton oxidation	5.0×10^{-4}	2.4×10^{-1}	3	30	-
Direct photolysis	-	-	3	30	UV/Vis (200-600 nm)
Photodegradation with H_2O_2	-	2.4×10^{-1}	3	30	UV/Vis (200-600 nm)
Photo-Fenton	5.0×10^{-4}	2.4×10^{-1}	3	30	UV/Vis (200-600 nm)

4.4.1.1 Fenton oxidation

The chromatograms obtained from the injection of different samples collected along the Fenton's reaction (at 0.5, 1, 2, 5, 8 and 24 h) are shown in Figure 4.17. Through the analysis of the chromatograms it is possible to verify that a rapid degradation of 5-Fu occurs for the first 30 min of reaction, which is reflected by the rapid decrease in peak area at t_r = 23.80 ± 0.02 min. Moreover, the decrease of 5-Fu peak area is followed by the increase of the area of peak 1 (t_r = 11 ± 0.02 min) until 2 hours of reaction, the time at which the area of peak 1 starts decreasing. Therefore, it can be concluded that the degradation of 5-Fu is followed by the formation of by-products and the degradation of by-products is a slower process since it is possible to verify its presence until 8 hours of reaction. These results are consistent with the results obtained in TOC, indicating that the Fenton process can mineralize only part of the 5-Fu by-products.

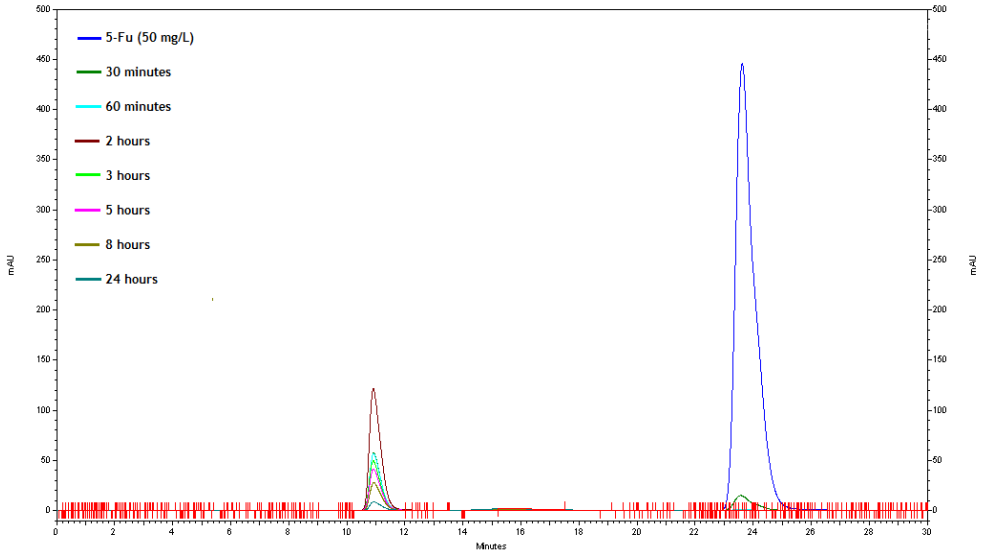


***Figure 4.17** Chromatograms with the elution profile of 5-Fu and elution profile of different samples collected along the reaction time at 30 and 60 min, 2, 3, 5, 8 and 24 hours.*

4.4.1.2 Direct photolysis

The chromatograms obtained from the injection of different samples collected along the direct photolysis (90 min, 2, 4, 6, and 8 hours) are presented in Figure 4.18. Through analysis of the chromatograms it is possible to verify that 5-Fu degradation is followed by the formation of different transformation products. Through the analysis of the chromatograms, it is possible to see that after the 5-Fu degradation (6 hours), the peak area of the degradation products decrease which indicates that the by-products are being oxidized. After 8 hours of reaction it is still possible to observe some degradation products. These results are concordant with the TOC analysis that indicate that after 8 hour of reaction the mineralization was only about 73% (Table 4.4).

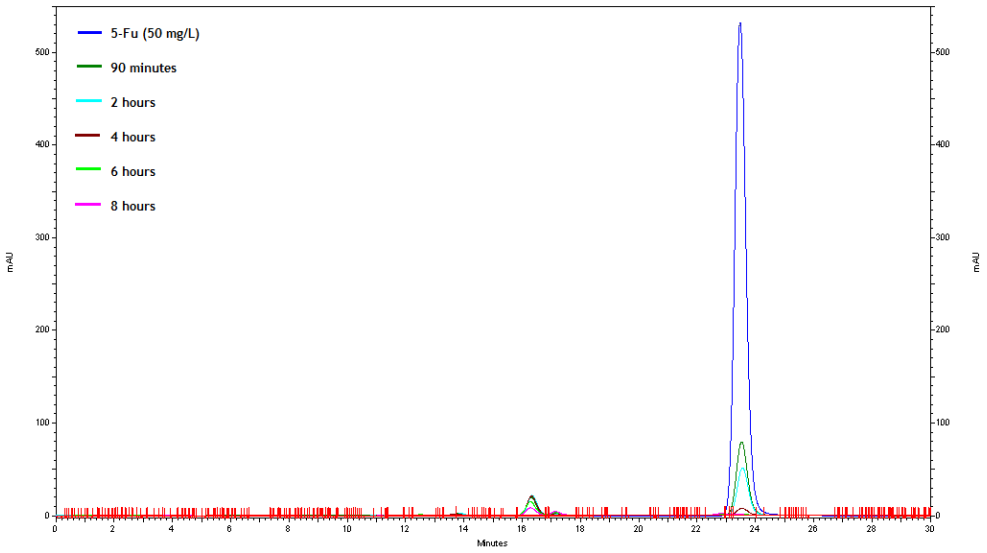


Figure 4.18 *Chromatograms with the elution profile of 5-Fu and different samples collected along the reaction time at 90 min, 2, 4, 6 and 8 hours.*

In Figure 4.19 is presented the magnified chromatogram of the samples collected along the direct photolysis process. Through analysis of the chromatograms is possible to observe the appearance of a larger number of peaks, when compared with the Fenton reaction, which indicates the formation of a greater number of degradation products. However, by-products formed along the photolysis are more easily degraded, because after 8 hour of reaction mineralization rate is higher than in Fenton process.

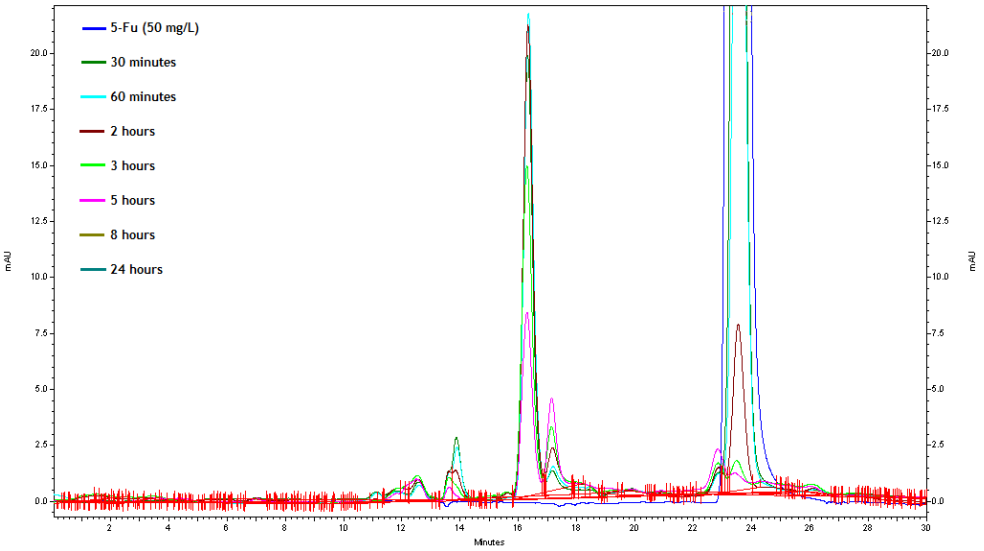


Figure 4.19 *Chromatograms with the elution profile of 5-Fu and different samples collected along the reaction time at 90 min, 2, 4, 6 and 8 hours.*

4.4.1.3 Photodegradation with H_2O_2

The chromatograms obtained from the injection of different samples collected along the photo-assisted degradation with H_2O_2 (10, 15 and 60 min, 2, 4 and 8 h) are shown in Figure 4.20. Analyzing the magnified chromatogram presented in Figure 4.21 is possible to see that after 8 hours there are no peaks in the chromatogram. These results are in agreement with the TOC results that indicate that after 8 hours of reaction all of 5-Fu was completely mineralized (Table 4.4).

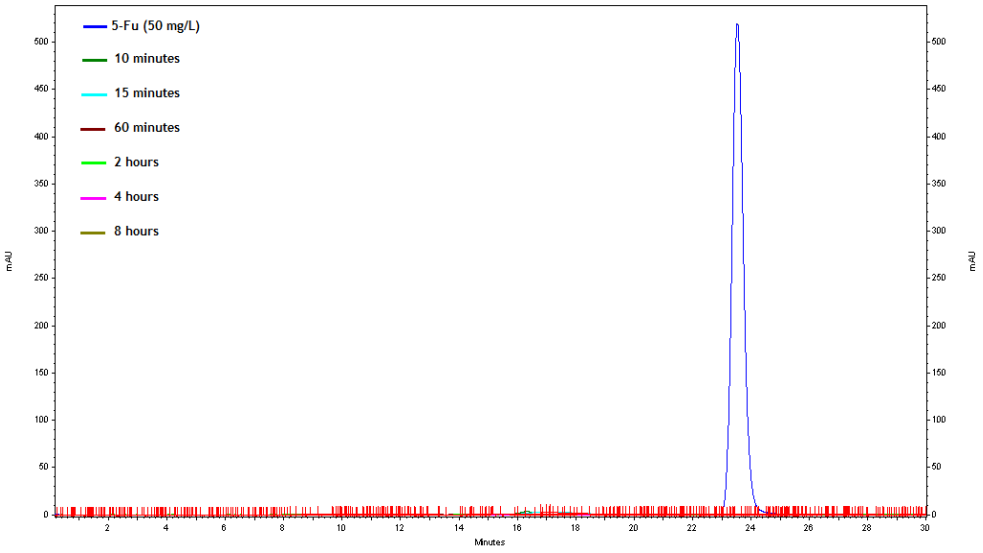


Figure 4.20 *Chromatograms with the elution profile of 5-Fu and different samples collected along the reaction time at 10, 15 and 60 min and 2,4 and 8 hours.*

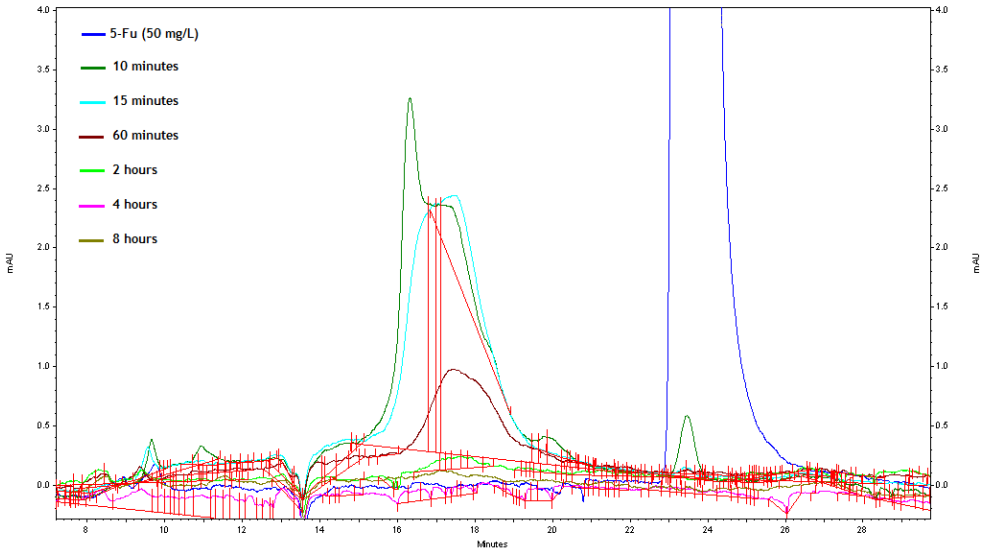


Figure 4.21 *Chromatograms with the elution profile of 5-Fu and different samples collected along the reaction time at 10, 15 and 60 min and 2,4 and 8 hours.*

4.4.1.4 Photo-Fenton process

The photo-Fenton process was the most effective method for 5-Fu mineralization; after 15 min of reaction 5-Fu and its degradation products were completely eliminated. In Figure 4.22 is presented the chromatograms obtained for 5-Fu and for a sample collected after 15 min of reaction. It is possible to observe that after 15 min, 5-Fu was completely eliminated and there are no peaks corresponding to degradation products. These results are in accordance with the TOC results that indicate that 5-Fu is completely mineralized after 15 minutes of reaction (Table 4.4).

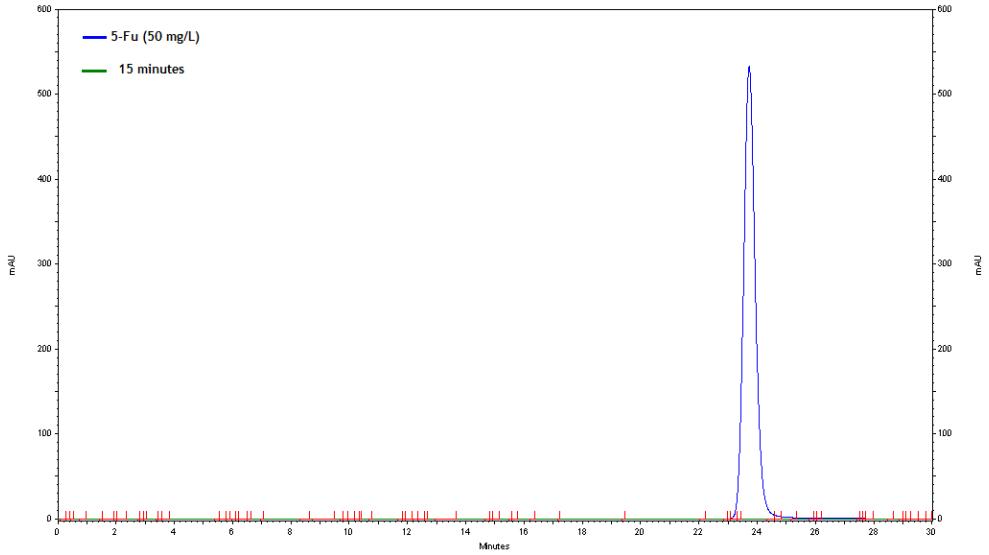


Figure 4.22 *Chromatograms with the elution profile of 5-Fu and a sample collected after 15 min of reaction.*

4.5 Toxicity

Due to its mode of action, practically all of the organisms are vulnerable to damage by 5-Fu, with teratogenecity being the greatest concern, even at low levels as 5 - 50 ng L^{-1}. Thus, despite all the methods used for degradation of 5-Fu have shown to be effective in its degradation (at least below the detection limit), it is necessary to study the toxicity of the compounds formed, since they can be toxic. Thus, in order to evaluate the toxicity of the parental compound and the transformation products a toxicity analysis was performed. The toxicity was assessed for a 5-Fu solution (50 mg L^{-1}) and a sample obtained after 8 hour of direct photolysis. The operation conditions are presented in Table 4.5. The results are present in Figure 4.23.

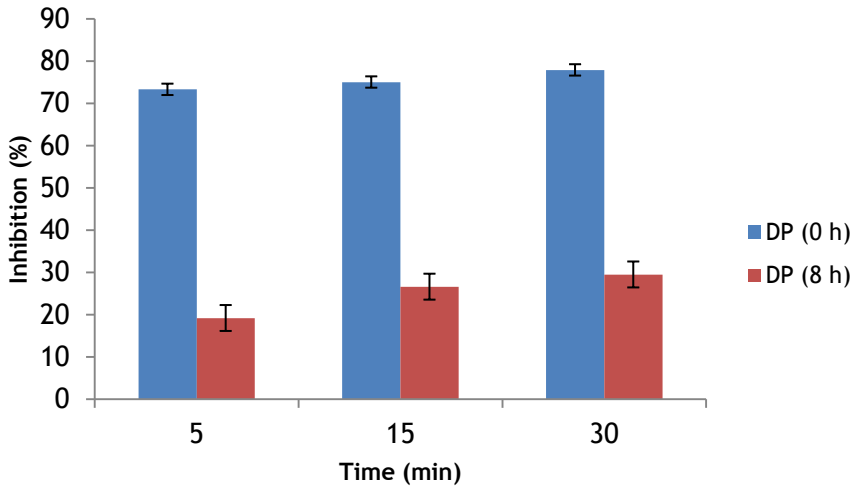


Figure 4.23 *Toxicity tests with V. fischeri (n = 2) for 0, 15 and 30 min of contact time with the bacteria for 0 hours and 8 hours of reaction in direct photolysis (DP).*

The toxicity analysis showed that the toxicity of the solution decreases after direct photolysis. Along the reactions different products are generated, and this results show that in directed photolysis the degradation products are less toxic than the parental compounds. However, more tests should be performed in order to evaluate the toxicity of these compounds, and the by-products formed along the other degradation processes.

5 Conclusions

Cytostatic drugs are substances widely used in treatment of cancer; among them the most commonly consumed is 5-Fu. Although the amount of 5-Fu in the aquatic environment may be still considered low, its continue input may constitute a long-term potential risk. Thus, since the removal of 5-Fu by conventional wastewater treatment is often incomplete and inefficient, is necessary to find alternatives for the current methods used for 5-Fu degradation.

In the present work it was investigated the application of different processes for 5-Fu removal: Fenton oxidation, direct photolysis, photo-oxidation with hydrogen peroxide and photo-Fenton. It was necessary to develop an analytical methodology to evaluate the 5-Fu degradation degree in samples from degradation experiments.

Based on the information described in the literature, a HPLC-DAD method for 5-Fu analysis was developed. The best performance of the analytical method was obtained at 25 °C, in isocratic conditions (0.2 mL min^{-1}) and with the following mobile phase: 97% water acidified with 0.01% of formic acid (v/v) and 3% methanol acidified with 0.01% of formic acid (v/v) . The limit of detection of this method was 0.006 mg L^{-1} and the limit of quantification was 0.02 mg L^{-1}. The method proved to be precise and accurate for the purpose that it was designed.

Fenton processes reveals to be an effective method for 5-Fu degradation. In the optimal conditions found 5-Fu was completed degraded after 2 hours of reaction; however, after 24 hour of reaction only about 50% of mineralization was achieved.

Direct photolysis treatment reveals to be effective on 5-Fu degradation, as after 4 hours of reaction 5-Fu was completely removed. Although 5-Fu was completely removed, after 8 hours of reaction only about 73% of 5-Fu was mineralized.

Similar results were obtained for 5-Fu degradation by photodegradation with hydrogen peroxide and photo-Fenton. 5-Fu was completely degraded after 2 min of reaction. However, different mineralization rates were obtained for photodegradation with hydrogen peroxide and photo-Fenton. The best mineralization was obtained in photo-Fenton process; after 15 min of reaction 5-Fu was completely mineralized.

Among the processes tested for 5-Fu degradation, the best performance was obtained for photo-Fenton process. Up to the author knowledge, photo-Fenton process (in the tested conditions) reveals to be more effective than the methods described is the literature, since none of the methods described got the total mineralization of the compound.

6 Limitations and Future work

Although good results were achieved and the main objectives of this work were met, there were some limitations, most of them associated with time and the availability of the equipments used.

The time of each analysis was long (around 30 minutes). Besides the fact the HPLC is share with other researchers, there were periods of time in which HPLC did not work due to technical problems, which leaded to a delay of the samples analysis.

Furthermore, initially there was no information in the literature about the 5-Fu degradation by Fenton and photo-Fenton processes, which led to the necessity to test some experimental parameters in ranges not know that could affect 5-Fu degradation processes and 5-Fu analysis by HPLC and TOC.

This work was a preliminary study about 5-Fu degradation by Fenton and photo-Fenton process, but much more can be done later on, especially regarding the photo-Fenton process. As future work I would like to suggest:

- To do a parametric study about photo-Fenton process in order to optimize the operating conditions such as catalyst concentration and hydrogen peroxide concentration, as well as the pH of the reaction; study the effect of different types of radiation and its intensity would be also relevant;
- To study the effect of the intensity of the radiation on photo-assisted degradation processes;
- To study the effect of pH on photodegradation processes with hydrogen peroxide;
- To study the degradation products and evaluate their toxicity;
- To study the 5-Fu degradation at lower concentrations in order to evaluate the process closer to the real conditions effluents;
- To study the process in a real effluent in order to evaluate its efficiency in the presence of other compounds.

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Appendix A - Analytical methods used for determination of 5-Fu

Table A 1 Analytical methods used for determination of 5-Fu in environmental matrices and more relevant characteristics of the analytical methodology employed

Analytical Method	Analyte	Sample type (Volume)	Separation	Detection	t_R (min)	Analytical parameters	Ref.
			HPLC				
LC-DAD	10 cytostatics including 5-Fu	Wastewater Surface water (n/m)	*Column:* C18 (250 x 4.0 mm; 5 µm); *Elution:* gradient; *Mobile phase:* (A) 0.01 mol L^{-1} KH_2PO_4 buffer (pH 3), (B) MeOH; *Flow-rate:* 1 mL min^{-1}; *Injection:* 20 µL.	UV 267 nm	5.1	*LOQ:* 50 µg L^{-1}; *RSD:* n/m;	[8]
			CE				
CE-UV	5-Fu	Hospital wastewater (50 mL)	*Capillary:* 56 cm x 75 µm (T = 40 °C); 30 kV at 100 µA; *Buffer:* 80% 160 mM sodium borate buffer (pH 9.5) + 20% ACN; *Injection:* 20 nL (30 mbar, 3.7 s).	UV 265 nm	t_M = 21	LOQ: 8.6 µg L^{-1}; RSD: 0.7–9.5%;	[41, 67]
			GC			NCI	
GC-MS	5-Fu and tamoxifen	Wastewater (150 mL)	*Column:* capillary RTX-5 (60 m x 0.25 mm, 0.25 µm); *Flow rate:* 1 mL min^{-1}; *Injection:* 1 µL on-column (85 °C).	MS *Ionization mode:* EI and NCI.	42.9	*LOQ:* 0.05 µg L^{-1}; *RSD:* 9%; EI *LOQ:* 0.09 µg L^{-1}; *RSD:* 9%;	[22]
			HPLC	UV 267 nm			
HPLC-MS/MS	5 compounds including 5-Fu	Hospital wastewater (50 mL)	*Cartridge:* ZIC-HILIC (150 x 2.1 mm, 3.5 µm); *Mobile phase:* (A) 30 nM ammonium acetate/ ACN (2/3, v/v), (B) ACN; *Flow-rate:* 0.2 mL min^{-1}; *Injection:* 2-6 µL.	QqQ MS *Ionization mode:* ESI(-). HRMS *Ionization mode:* ESI(-).	5.9	*LOQ:* 0.005 µg L^{-1}; *RSD:* 1.7 – 5.6%;	[68]
			HPLC				
LC-DAD	5-Fu and 5-Bu	Surface sample (2 mL)	*Cartridge:* C18 (250 x 4.6 mm, 5 µm); *Mobile phase*: (A) 20 mM ammonium acetate buffer pH 4.7, (B) MeOH (95:5 v/v); *Flow rate:* 1.2 mL min^{-1}; *Injection:* 500 µL.	HPLC-DAD 265 nm	4.5	*LOQ:* 25 µg L^{-1}L *RSD:* 2.1 – 14%	[24]

Table A 1 Analytical methods used for determination of 5-Fu in environmental matrices and more relevant characteristics of the analytical methodology employed (continued)

Analytical Method	Analyte	Sample type (Volume)	Separation	Detection	t_R (min)	Analytical parameters	Ref.
GC-MS/MS	5-Fu	Wastewater Surface water (100 mL)	**GC** *Column:* Capillary column (30 m x 0.25 mm x 0.25 µm); *Injection:* 1 µL on-column (250 °C).	Ion trap MS *Ionization mode:* EI	9.7	SW *LOQ:* 0.00054 µg L^{-1} *RSD:* 5.1–6.7% WW *LOQ:* 0,0016 µg L^{-1} RSD: 5.2 – 14%	[9]
LC-MS/MS	Capecitabine transformations products and 5-Fu transformations products	Wastewater (n/m)	**UPLC** *Cartridge:* C18 (5 cm x 2.1 mm, 1.7 µm); *Mobile phase:* (A) 0.01% acid formic, (B) ACN; *Flow rate:* 0.3 mL min^{-1}; *Injection:* 7.5 µL; *Temperature*: EI(+): 50 °C; EI(-): 30 °C.	MS QqToF *Ionization mode:* EI(+) and EI(-) HRMS	0.72	*LOQ:* n/m RSD: n/m	[9]
LC-MS/MS	5-Fu and cyclophospha-mide	Water (n/m)	**HPLC** *Cartridge:* C18 (150 x 4.6 mm, 5 µm) *Mobile phase:* (A) 0.1% formic acid in water; (B) 0.1% formic acid in 100% MeOH. *Flow rate:* 1 mL min^{-1}; *Injection:* 20 µL.	MS *Ionization mode:* EI(-)	3.7±(30%)	*LOQ:* n/m *RSD:* n/m	[15]
LC-MS/MS	5-Fu	Wastewater (n/m)	**HPLC** *Cartridge:* C18 (150 x 4.6 mm, 5 µm); *Mobile phase:* (A) 0.1% formic acid in water; (B) 0.1% formic acid in 100% MeOH; *Flow rate:* 1 mL min^{-1}; *Injection:* 20 µL.	MS/MS Ionization mode: EI(-)	3.7±(30%)	*LOQ:* n/m *RSD:* n/m	[48]
GC-MS/MS	5-Fu	Hospital wastewater (100 mL)	**GC** *Column:* Factor Four 5-ms capillary column (30 m× 0.25 mm i.d.×0.25) *Flow rate:* 1 mL min^{-1}; *Injection:* 2 µL on-column (250 ºC). **HPLC-DAD** *Column:* C18 (150 x 4.6 mm; 5 µm); *Mobile phase:* Water:ACN. *Flow-rate:* 1 mL/min; *Injection:* 10 µL	MS/MS MRM DAD 265 nm	4.0	*LOQ:* 40 µg L^{-1} *RSD:* <10%	[23]

Table A 1 Analytical methods used for determination of 5-Fu in environmental matrices and more relevant characteristics of the analytical methodology employed (continued)

Analytical Method	Analyte	Sample type (Volume)	Separation	Detection	t_R (min)	Analytical parameters	Ref.
			HPLC				
LC-PDA	5-Fu	Polymeric nanoparticles (n/m)	*Cartridge:* C18 (250 x 4.6 mm, 5 µm) *Mobile phase:* ACN:Water; *Flow rate:* 1 mL min^{-1}; *Injection:* 100 µL.	PDA 265 nm	3.5	*LOQ*: 32.78 ng L^{-1} *RSD:* 1.49%	[25]
			HPLC				
LC-UV	5-Fu, uracil, and thymine	Human plasma (n/m)	*Column:* C18 (300 x 3.9 mm; 10 µm); *Mobile phase:* MeOH:Water (10:90, v/v); *Flow-rate:* 1 mL min^{-1}; *Injection:* 20 µL.	UV 260 nm	4.5	*LOQ*: 30 ng mL^{-1} *RSD: n/m*	[26]
			HPLC				
LC-UV	5-Fu and Tegafur	Beagle dog plasma (n/m)	*Column:* C18 (250 x 3.6 mm; 5 µm); *Mobile phase:* 10 mM acetic acid: MeOH (90:10 (v/v)); *Flow-rate:* 1 mL min^{-1}; *Injection:* 20 µL.	UV 260 nm	4.5	*LOQ:* 4.8 µg L^{-1} *RSD: <15%*	[27]
			HPLC				
LC-UV	5-Fu	Human plasma (n/m)	*Column:* C18 (250 x 4.6 mm; 5 µm); *Mobile phase:* (A) potassium phosphate (pH 5.5; 0.01 M); (B) MeOH:potassium phosphate (pH 5.5; 0.01 M) (50:50, v/v) *Flow-rate:* 1.20 mL min^{-1}; *Injection:* 120 µL.	UV 266 nm	7.5	*LOQ:* 4.8 µg L^{-1} *RSD:1.9-2.7%*	[69]
			HPLC				
LC-UV	4 compounds including 5-Fu	Human plasma (n/m)	*Column:* C18 (250x 4.6 mm; 5 µm); *Mobile phase:* (A) 0.05 M disodium hydrogenphosphate in 0.1% (w/v) aqueous sodium laurylsulfate and acetonitrile (1:1); (B) 0.02 M sodium dihydrogenphosphate aqueous solution and acetonitrile (7:3); *Flow-rate:* 1 mL min^{-1}; *Injection:* 20 µL.	UV 250 nm (mobile phase A) UV 260 nm (mobile phase B)	3.0	*LOQ:* 1.6 µg mL^{-1} *RSD: <10%*	[28]
			HPLC				
LC-UV	5-Fu	Human plasma (150 µL)	*Column:* Aminex HPX-87H (300×7.8 mm, 9 µm); *Mobile phase:* 0.005 M sulfuric acid *Flow-rate:* 0.5 mL min^{-1}; *Injection:* 50 µL.	UV 265 nm	24	*LOQ:* 25 µg mL^{-1} *RSD: %*	[29]
			HPLC				
LC-Fluorescence	5-Fu and FdUMP	Tissue and serum (n/m)	*Column:* C18 (250 × 30 mm, 10 µm); *Mobile phase:* (A) water/MeOH with 1% of acetic acid; (B) PBS/MEOH (85:15); *Flow-rate*:1 mL min^{-1}; *Injection:* 30 µL.	Fluorescence Excitation: 266 nm; Emission: 350 nm.	A: 3.5; B: 2.5;	*LOQ:* 50 pg *RSD: n/m*	[70]

Table A 1 Analytical methods used for determination of 5-Fu in environmental matrices and more relevant characteristics of the analytical methodology employed (continued)

Analytical Method	Analyte	Sample type (Volume)	Separation	Detection	t_R (min)	Analytical parameters	Ref.
			HPLC				
LC-MS/MS	5-Fu, 5-Furd, 5-FdUrd, 5-FdUMP, dUMP and TMP	Cultured cell (n/m)	*Column:* C18 (100 mm × 2.1 mm, 3.5 µm); *Mobile phase:* ammonium formate buffer (5 mM, pH 4)/MeOH/water (5/5/90, v/v) *Flow-rate:* 0,2 mL min^{-1}; *Injection:* 10 µL.	MS/MS	3,22	*LOQ:* n/m *RSD: n/m*	[30]
			HPLC				
LC-UV	5-Fu and its metabolites	Colon cancer patients (n/m)	*Column:* Spherisorb™ ODS1 not end-capped column (250× 4,6 mm, 5 µm) *Mobile phase:* 0.015 M K_3PO_4 buffer (pH 5) *Flow-rate:* 1 mL min^{-1}; *Injection:* µL.	UV 210 nm	n/M	*LOQ:* 0.5 µg L^{-1} *RSD: n/m*	[71]
			HPLC				
LC-UV	5-Fu	Human plasma (n/m)	*Column:* C18 (5 µm, 250×4.0 mm); *Mobile phase:* potassium dihydrogenphosphate (0.05 *M*, adjusted to pH 3 with 85% orthophosphoric acid); *Flow-rate:* 1,7 mL min^{-1}; *Injection:* 20 µL.	UV 266 nm	7	*LOQ:* 2 µg mL^{-1} *RSD: n/m*	[31]
			HPLC				
LC-UV	5-Fu	Human plasma (n/m)	*Column:* Inertsil ODS-3 column (250×4.6 mm ID; 5 µ*M* particle size) *Mobile phase:* water with the pH adjusted to pH 2.0 (perchloric acid) *Flow-rate*:1 mL min^{-1};	UV 266 nm		*LOQ:* 0.20 µg L^{-1} *RSD:* n/m	[32]
			HPLC				
LC-UV	5-Fu	Human plasma (n/m)	*Column:* C18 µBondapak columns (10 µm, 125 Å, 300 × 3.9 mm) *Mobile phase:* 1% ACN in 20 m*M* acetic acid. *Flow-rate:* 0.9 mL min^{-1}; *Injection:* 100 µL.	UV 266 nm	16	*LOQ:* n/m *RSD:* n/m	[33]

n/m: not mentioned; n/a: not applied; LC: liquid chromatography; MS: mass spectrometry; QqQ: triple quadrupole; QqToF: quadrupole orthogonal acceleration time-of-flight mass spectrometer; UPLC: Ultraperformance liquid chromatography; LOQ: limit of quantification; RSD: relative standard deviation; HPLC: High performance liquid chromatography; N-VPDVB: N-vinylpyrrolidone-divinylbezene; MeOH: methanol; ACN: Acetonitrila; t_R: retention time; t_M: migration time; HILIC: hydrophilic interaction liquid chromatography; EI: electron impact or electron ionization; NCI: negative chemical ionization; ESI: electrospray ionization; DAD: diode array detection; HRMS: High resolution mass spectrometry; MRM: multiple reaction monitoring; *GC: gas chromatography; PDA: photodiode array detector*.

Appendix B - Method development

To increase 5-Fu's retention time, in order to perform a better separation between 5-Fu and any degradation products formed along the reaction, it was necessary to optimize some experimental parameters to improve the analytical method. The effect of composition of mobile phases, temperature, pH, and flow rate was studied. The results are presented in Table B 1.

Table B 1 Method development: influence of mobile phase, pH, temperature and flow rate in 5-Fu analysis by HPLC-DAD

Parameter	Mobile phase A	% A (v/v)	Mobile phase B	% B (v/v)	Acid	% Acid (v/v)	Temperature (°C)	Flow rate (ml min^{-1})	Area (a.u.)	Retention time (min)
Mobile phase	Water	97	Methanol	3	Formic acid	0.01	25	1	201788	4.633
	Water	95	Methanol	5	Formic acid	0.01	25	1	218282	4.100
	Water	70	Methanol	30	Formic acid	0.01	25	1	201793	2.493
pH	Water	95	Methanol	5	Formic acid	0.01	25	1	218282	4.100
	Water	95	Methanol	5	Formic acid	0.1	25	1	192994	4.100
	Phosphate buffer	97	Methanol	3	-	0	25	1	223132	4.637
	Water	97	Methanol	3	-	0	25	1	182390	4.617
	Water	97	Methanol	3	Acetic acid	0.01	25	1	200513	4.657
Temperature	Water	95	Methanol	5	Formic acid	0.1	35	1	216556	3.733
	Water	95	Methanol	5	Formic acid	0.1	25	1	192994	4.100
	Water	95	Methanol	5	Formic acid	0.1	15	1	147595	4.667
	Water	95	Methanol	5	Formic acid	0.1	10	1	69158	5.093
Flow rate	Water	97	Methanol	3	Formic acid	0.01	25	1	200513	4.657
	Water	97	Methanol	3	Formic acid	0.01	25	0.75	309148	6.220
	Water	97	Methanol	3	Formic acid	0.01	25	0.5	495964	9.367
	Water	97	Methanol	3	Formic acid	0.01	25	0.2	1266261	23.464